

**METHODS AND COMPOSITIONS FOR CONTROL OF BONE FORMATION VIA  
MODULATION OF LEPTIN ACTIVITY**

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**1     INTRODUCTION**

The present invention relates to compositions and methods for the treatment, diagnosis and prevention of conditions, disorders or diseases involving bone, including, but not limited to, osteoporosis. The invention relates to modulation of the receptor signaling pathway for the polypeptide hormone leptin. More particularly the present invention relates to the modulation of leptin synthesis, leptin receptor synthesis, leptin binding to its receptor, and leptin signaling to bone cells.

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The present invention also provides methods for the identification and prophylactic or therapeutic use of compounds in the treatment, prognosis and diagnosis of conditions, disorders, or diseases involving bone. Additionally, methods are provided for the diagnostic monitoring of patients undergoing clinical evaluation for the treatment of conditions or disorders involving bone, for monitoring the efficacy of compounds in clinical trials and for identifying subjects who may be predisposed to such conditions, disorders, or diseases involving bone.

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**2     BACKGROUND OF THE INVENTION**

The physiological process of bone remodeling allows constant renewal of bone through two well-defined sequential cellular processes. Karsenty, 1999, Genes and Development, 13:3037-3051. The initial event is resorption of preexisting bone by the osteoclasts, followed by de novo bone formation by the osteoblasts. These two processes in bone remodeling must maintain equilibrium of bone mass within narrow limits between the

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end of puberty and the arrest of gonadal function. The molecular mechanisms responsible for maintaining a constant bone mass are unknown, yet several lines of evidence suggest that this may be achieved, at least in part, through a complex endocrine regulation. For example, gonadal failure and the concomitant deficiency of the sex steroids stimulates the bone resorption process of bone remodeling and eventually leads to osteopenia (low bone mass) or osteoporosis (low bone mass and high susceptibility to fractures). Likewise, the recent identification of osteoprotegerin in serum and its functional characterization through a systemic route is another indication that secreted molecules affect osteoclastic bone resorption. Simonet *et al.*, 1997, Cell, 89:309-319. This systemic control of bone resorption suggests that other circulating molecules, yet to be identified, could control bone formation via the osteoblasts. The identification of these hormones or growth factors, if they exist, is of paramount importance given the incidence and morbidity of diseases affecting bone remodeling.

One such disease is osteoporosis. Riggs *et al.*, 1998, J. Bone Miner. Res., 13:763-773. Osteoporosis is the most common disorder affecting bone remodeling and the most prevalent disease in the Western hemisphere. At the physiopathological level, hallmarks of the disease are that bones exhibit a lowered mass, that is, are less dense and, thus, subject to fractures. In addition, the onset of osteoporosis in both sexes is intimately linked to arrest of gonadal function and is rarely observed in obese individuals. At the cellular level, osteoporosis is characterized by a loss in equilibrium of bone remodeling favoring bone resorption over bone formation, which leads to the lowered bone mass and increased bone fractures. At the molecular level, the pathogenesis of osteoporosis remains largely unknown.

### **3 SUMMARY OF THE INVENTION**

An object of the present invention is the treatment, diagnosis and/or prevention of bone disease through manipulation of the leptin signaling pathway. Bone diseases which can be treated and/or prevented in accordance with the present invention include bone diseases characterized by a decreased bone mass relative to that of corresponding non-diseased bone, including, but not limited to osteoporosis, osteopenia and Paget's disease. Bone diseases which can be treated and/or prevented in accordance with the present invention also include

bone diseases characterized by an increased bone mass relative to that of corresponding non-diseased bone, including, but not limited to osteopetrosis, osteosclerosis and osteochondrosis.

Thus, in accordance with one aspect of the present invention, there is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound that lowers leptin level in blood serum, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin synthesis or increase leptin breakdown. Among such compounds are antisense, ribozyme or triple helix sequences of a leptin-encoding polypeptide.

In accordance with another aspect of the present invention, there is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound that lowers leptin level in cerebrospinal fluid, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin synthesis or increase leptin breakdown, and compounds that bind leptin in blood.

Particular embodiments of the methods of the invention include, for example, a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone, and wherein the compound is selected from the group consisting of compounds which bind leptin in blood, including, but not limited to such compounds as an antibody which specifically binds leptin, a soluble leptin receptor polypeptide, an inter-alpha-trypsin inhibitor heavy chain related protein and an alpha 2-macroglobulin protein.

In accordance with another aspect of the present invention, there is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound that lowers the level of phosphorylated Stat3 polypeptide, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin synthesis or

increase leptin breakdown, compounds that bind leptin in blood, and leptin receptor antagonist compounds, such as acetylphenol compounds, antibodies which specifically bind leptin, antibodies which specifically bind leptin receptor, and compounds that comprise soluble leptin receptor polypeptide sequences.

5 In accordance with another aspect of the present invention, there is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound that lowers leptin receptor levels in hypothalamus, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin receptor synthesis or increase leptin receptor breakdown. Among such compounds are antisense, ribozyme or triple helix sequences of a leptin receptor-encoding polypeptide.

10 In accordance with yet another aspect of the present invention, there is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound that increases leptin level in blood serum and/or cerebrospinal fluid, wherein the bone disease is characterized by a increased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that increase or induce leptin synthesis or decrease leptin breakdown.

15 In accordance with another aspect of the present invention, there is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound that increases the level of phosphorylated Stat3 polypeptide, wherein the bone disease is characterized by a increased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that increase or induce leptin synthesis or decrease leptin breakdown, and leptin receptor agonist compounds.

20 In accordance with another aspect of the present invention, there is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound that increases leptin receptor levels in hypothalamus, wherein the bone disease is characterized by a increased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds

and methods include, but are not limited to ones that increase or induce leptin receptor synthesis or decrease leptin receptor breakdown.

In accordance with yet another aspect of the present invention, there is a method of preventing a bone disease comprising: administering to a mammal at risk for the disease a compound that lowers leptin level in blood serum, at a concentration sufficient to prevent the bone disease, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin synthesis or increase leptin breakdown. Among such compounds are antisense, ribozyme or triple helix sequences of a leptin-encoding polypeptide.

In accordance with another aspect of the present invention, there is a method of preventing a bone disease comprising: administering to a mammal at risk for the bone disease a compound that lowers leptin level in cerebrospinal fluid, at a concentration sufficient to prevent the bone disease, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin synthesis or increase leptin breakdown, and compounds that bind leptin in blood.

Particular embodiments of the methods of the invention include, for example, a method of preventing a bone disease comprising: administering to a mammal at risk for the bone disease a compound at a concentration sufficient to prevent the bone disease, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone, and wherein the compound is selected from the group consisting of compounds which bind leptin in blood, including, but not limited to such compounds as an antibody which specifically binds leptin, a soluble leptin receptor polypeptide, an inter-alpha-trypsin inhibitor heavy chain related protein and an alpha 2-macroglobulin protein.

In accordance with another aspect of the present invention, there is a method of preventing a bone disease comprising: administering to a mammal at risk for the bone disease a compound that lowers the level of phosphorylated Stat3 polypeptide, at a concentration sufficient to prevent the bone disease, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific

embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin synthesis or increase leptin breakdown, compounds that bind leptin in blood, and leptin receptor antagonist compounds, such as acetylphenol compounds, antibodies which specifically bind leptin, antibodies which specifically bind leptin receptor, and compounds that comprise soluble leptin receptor polypeptide sequences.

In accordance with another aspect of the present invention, there is a method of preventing a bone disease comprising: administering to a mammal at risk for the bone disease a compound that lowers leptin receptor levels in hypothalamus, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin receptor synthesis or increase leptin receptor breakdown. Among such compounds are antisense, ribozyme or triple helix sequences of a leptin receptor-encoding polypeptide.

In accordance with yet another aspect of the present invention, there is a method of preventing a bone disease comprising: administering to a mammal at risk for the bone disease a compound that increases leptin level in blood serum and/or cerebrospinal fluid, at a concentration sufficient to prevent the bone disease, wherein the bone disease is characterized by a increased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that increase or induce leptin synthesis or decrease leptin breakdown.

In accordance with another aspect of the present invention, there is a method of preventing a bone disease comprising: administering to a mammal at risk for the bone disease a compound that increases the level of phosphorylated Stat3 polypeptide, at a concentration sufficient to prevent the bone disease, wherein the bone disease is characterized by a increased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that increase or induce leptin synthesis or decrease leptin breakdown, and leptin receptor agonist compounds.

In accordance with another aspect of the present invention, there is a method of preventing a bone disease comprising: administering to a mammal at risk for the disease a compound that increases leptin receptor levels in hypothalamus, at a concentration sufficient

to prevent the bone disease, wherein the bone disease is characterized by a increased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that increase or induce leptin receptor synthesis or decrease leptin receptor breakdown.

5 In accordance with yet another aspect of the present invention, there is a method of diagnosing or prognosing a bone disease in a mammal, such as a human, comprising:

- (a) measuring leptin levels in blood serum of a mammal, *e.g.*, a mammal suspected of exhibiting or being at risk for the bone disease; and
- (b) comparing the level measured in (a) to the leptin level in control blood serum,

10 so that if the level obtained in (a) is higher than that of the control, the mammal is diagnosed or prognosed as exhibiting or being at risk for the bone disease, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone.

15 In accordance with another aspect of the present invention, there is a method of diagnosing or prognosing a bone disease in a mammal, such as a human, comprising:

- (a) measuring leptin levels in cerebrospinal fluid of a mammal, *e.g.*, a mammal suspected of exhibiting or being at risk for the bone disease; and
- (b) comparing the level measured in (a) to the leptin level in control cerebrospinal fluid,

20 so that if the level obtained in (a) is higher than that of the control, the mammal is diagnosed as exhibiting or being at risk for the bone disease, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone.

25 In accordance with yet another aspect of the present invention, there is a method of diagnosing or prognosing a bone disease in a mammal, such as a human, comprising:

- (a) measuring leptin levels in blood serum of a mammal, *e.g.*, a mammal suspected of exhibiting or being at risk for the bone disease; and
- (b) comparing the level measured in (a) to the leptin level in control blood serum,

so that if the level obtained in (a) is lower than that of the control, the mammal is diagnosed as exhibiting or being at risk for the bone disease, wherein the bone disease is characterized by an increased bone mass relative to that of corresponding non-diseased bone.

In accordance with another aspect of the present invention, there is a method of  
5 diagnosing or prognosing a bone disease in a mammal, such as a human, comprising:

- (a) measuring leptin levels in cerebrospinal fluid of a mammal, *e.g.*, a mammal suspected of exhibiting or being at risk for the bone disease; and
- (b) comparing the level measured in (a) to the leptin level in control  
10 cerebrospinal fluid,

so that if the level obtained in (a) is lower than that of the control, the mammal is diagnosed as exhibiting or being at risk for the bone disease, wherein the bone disease is characterized by an increased bone mass relative to that of corresponding non-diseased bone.

In accordance with yet another aspect of the present invention, there is a method of  
15 monitoring efficacy of a compound for treating a bone disease in a mammal, such as a human, comprising:

- (a) administering the compound to a mammal;
- (b) measuring leptin levels in blood serum of the mammal; and
- (c) comparing the level measured in (b) to the leptin level in blood serum  
20 of the mammal prior to administering the compound,

thereby monitoring the efficacy of the compound, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone.

In accordance with another aspect of the present invention, there is a method of  
25 monitoring efficacy of a compound for treating a bone disease in a mammal, such as a human, comprising:

- (a) administering the compound to a mammal;
- (b) measuring leptin levels in cerebrospinal fluid of the mammal; and
- (c) comparing the level measured in (b) to the leptin level in cerebrospinal  
fluid of the mammal prior to administering the compound,

30 thereby monitoring the efficacy of the compound, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone.

In accordance with yet another aspect of the present invention, there is a method of monitoring efficacy of a compound for treating a bone disease in a mammal, such as a human, comprising:

- (a) administering the compound to a mammal;
- (b) measuring leptin levels in blood serum of the mammal; and
- (c) comparing the level measured in (b) to the leptin level in blood serum of the mammal prior to administering the compound,

thereby monitoring the efficacy of the compound, wherein the bone disease is characterized by a increased bone mass relative to that of corresponding non-diseased bone.

In accordance with another aspect of the present invention, there is a method of monitoring efficacy of a compound for treating a bone disease in a mammal, such as a human, comprising:

- (a) administering the compound to a mammal;
- (b) measuring leptin levels in cerebrospinal fluid of the mammal; and
- (c) comparing the level measured in (b) to the leptin level in cerebrospinal fluid of the mammal prior to administering the compound,

thereby monitoring the efficacy of the compound, wherein the bone disease is characterized by a increased bone mass relative to that of corresponding non-diseased bone.

In accordance with another aspect of the present invention, there is a method for identifying a compound to be tested for an ability to modulate (increase or decrease) bone mass in a mammal, comprising:

- (a) contacting a test compound with a polypeptide; and
- (b) determining whether the test compound binds the polypeptide, so that if the test compound binds the polypeptide, then a compound to be tested for an ability to modulate bone mass is identified, wherein the polypeptide is selected from the group consisting of a leptin polypeptide and a leptin receptor polypeptide.

In accordance with another aspect of the present invention, there is a method for identifying a compound that modulates (increases or decreases) bone mass in a mammal, comprising:

- (a) contacting test compounds with a polypeptide;
- (b) identifying a test compound that binds the polypeptide; and

(c) administering the test compound in (b) to a non-human mammal, and determining whether the test compound modulates bone mass in the mammal relative to that of a corresponding bone in an untreated control non-human mammal, wherein the polypeptide is selected from the group consisting of a leptin polypeptide and a leptin receptor polypeptide, so that if the test compound modulates bone mass, then a compound that modulates bone mass in a mammal is identified.

In accordance with yet another aspect of the present invention, there is a method for identifying a compound to be tested for an ability to modulate (increase or decrease) bone mass in a mammal, comprising:

(a) contacting a test compound with a leptin polypeptide and a leptin receptor polypeptide for a time sufficient to form leptin/leptin receptor complexes; and

(b) measuring leptin/leptin receptor complex level, so that if the level measured differs from that measured in the absence of the test compound, then a compound to be tested for an ability to modulate bone mass is identified.

In accordance with another aspect of the present invention, there is a method for identifying a compound to be tested for an ability to decrease bone mass in a mammal, comprising:

(a) contacting a test compound with a cell which expresses a functional leptin receptor; and

(b) determining whether the test compound activates the leptin receptor, wherein if the compound activates the leptin receptor a compound to be tested for an ability to decrease bone mass in a mammal is identified.

In accordance with another aspect of the present invention, there is a method for identifying a compound that decreases bone mass in a mammal, comprising:

(a) contacting a test compound with a cell that expresses a functional leptin receptor, and determining whether the test compound activates the leptin receptor;

(b) administering a test compound identified in (a) as activating the leptin receptor to a non-human animal, and determining whether the test compound decreases bone mass of the animal relative to that of a corresponding bone of a control non-human animal, so that if the test compound decreases bone mass, then a compound that decreases bone mass in a mammal is identified.

In accordance with another aspect of the present invention, there is a method for identifying a compound to be tested for an ability to increase bone mass in a mammal, comprising:

(a) contacting a leptin polypeptide and a test compound with a cell that expresses a functional leptin receptor; and

(b) determining whether the test compound lowers activation of the leptin receptor relative to that observed in the absence of the test compound; wherein a test compounds that lowers activation of the leptin receptor is identified as a compound to be tested for an ability to increase bone mass in a mammal.

In accordance with yet another aspect of the present invention, there is a method for identifying a compound that increases bone mass in a mammal, comprising:

(a) contacting a leptin polypeptide and a test compound with a cell that expresses a functional leptin receptor, and determining whether the test compound decreases activation of the leptin receptor;

(b) administering a test compound identified in (a) as decreasing leptin receptor to a non-human animal, and determining whether the test compound increases bone mass of the animal relative to that of a corresponding bone of a control non-human animal, so that if the test compound increases bone mass, then a compound that increases bone mass in a mammal is identified.

The present invention also provides pharmaceutical compositions which can be used to treat and/or prevent bone diseases.

Other and further objects, features and advantages would be apparent and eventually more readily understood by reading the following specification and by reference to the accompanying drawings forming a part thereof, or any examples of the presently preferred embodiments of the invention are given for the purpose of the disclosure.

### **3.1 Definitions**

The following terms used herein shall have the meaning indicated:

Leptin, ("Ob") as used herein, is defined by the endogenous polypeptide product of an *ob* gene, preferably a human *ob* gene, of which the known activities are mediated through the hypothalamus.

Leptin receptor ("ObR"), as used herein, is defined by the receptor through which the leptin hormone binds to generate its signal; preferably, this term refers to a human leptin receptor.

Bone disease, as used herein, refers to any bone disease or state which results in or is characterized by loss of health or integrity to bone and includes, but is not limited to, osteoporosis, osteopenia, faulty bone formation or resorption, Paget's disease, fractures and broken bones, bone metastasis, osteopetrosis, osteosclerosis and osteochondrosis. More particularly, bone diseases which can be treated and/or prevented in accordance with the present invention include bone diseases characterized by a decreased bone mass relative to that of corresponding non-diseased bone (*e.g.*, osteoporosis, osteopenia and Paget's disease), and bone diseases characterized by an increased bone mass relative to that of corresponding non-diseased bone (*e.g.*, osteopetrosis, osteosclerosis and osteochondrosis). Prevention of bone disease includes actively intervening as described herein prior to onset to prevent the disease. Treatment of bone disease encompasses actively intervening after onset to slow down, ameliorate symptoms of, or reverse the disease or situation. More specifically, treating, as used herein, refers to a method that modulates bone mass to more closely resemble that of corresponding non-diseased bone (that is a corresponding bone of the same type, *e.g.*, long, vertebral, *etc.*) in a non-diseased state.

Leptin receptor antagonist, as used herein, refers to a factor which neutralizes or impedes or otherwise reduces the action or effect of a leptin receptor. Such antagonists can include compounds that bind leptin or that bind leptin receptor. Such antagonists can also include compounds that neutralize, impede or otherwise reduce leptin receptor output, that is, intracellular steps in the leptin signaling pathway following binding of leptin to the leptin receptor, *i.e.*, downstream events that affect leptin/leptin receptor signaling, that do not occur at the receptor/ligand interaction level. Leptin receptor antagonists may include, but are not limited to proteins, antibodies, small organic molecules or carbohydrates, such as, for example, acetylphenol compounds, antibodies which specifically bind leptin, antibodies which specifically bind leptin receptor, and compounds that comprise soluble leptin receptor polypeptide sequences.

Leptin receptor agonist, as used herein, refers to a factor which activates, induces or otherwise increases the action or effect of a leptin receptor. Such agonists can include

compounds that bind leptin or that bind leptin receptor. Such antagonists can also include compounds that activate, induce or otherwise increase leptin receptor output, that is, intracellular steps in the leptin signaling pathway following binding of leptin to the leptin receptor, *i.e.*, downstream events that affect leptin/leptin receptor signaling, that do not occur at the receptor/ligand interaction level. Leptin receptor agonists may include, but are not limited to proteins, antibodies, small organic molecules or carbohydrates, such as, for example, leptin, leptin analogs, and antibodies which specifically bind and activate leptin.

An agent is said to be administered in a "therapeutically effective amount" if the amount administered results in a desired change in the physiology of a recipient mammal, *e.g.*, results in an increase or decrease in bone mass relative to that of a corresponding bone in the diseased state; that is, results in treatment, *i.e.*, modulates bone mass to more closely resemble that of corresponding non-diseased bone (that is a corresponding bone of the same type, *e.g.*, long, vertebral, *etc.*) in a non-diseased state.

ECD, as used herein, refers to extracellular domain.

TM, as used herein, refers to transmembrane domain.

CD, as used herein, refers to cytoplasmic domain.

#### **4 BRIEF DESCRIPTION OF THE FIGURES**

**Figures 1A-1F.** High bone mass phenotype in ob/ob and db/db mice. In Figure 1A, an X-ray analysis of vertebrae (vert.) and long bones (femurs) of 6 month-old wild-type (wt) and ob/ob mice is shown. Figure 1B demonstrates a histological analysis of bones of 3 month-old (3m.) and 6 month-old (6m.) wt and ob/ob mice. The two upper panels demonstrate analysis of vertebrae, and the two bottom panels demonstrate long bones. Mineralized bone matrix is stained in black by the von Kossa reagent. Figure 1C shows quantification of the increase in bone volume in ob/ob mice. BV/TV, bone volume over trabecular volume. Grey bars, wt mice; black bars, ob/ob mice. Figure 1D illustrates a three points bending analysis of femur from wild-type (wt), ob/ob and wild-type ovariectomized (wt-OVX) mice. Figure 1E is a histological analysis of vertebrae of 6 month-old wt and db/db mice. Figure 1F is a quantification of the increase in bone volume in db/db mice. Asterisks indicate a statistically significant difference between two groups of mice ( $p < 0.05$ ). Error bars represent standard error of the mean (SEM).

**Figure 2A-2D.** High bone mass phenotype of the ob/ob mice is due to leptin deficiency, not to obesity. Figure 2A demonstrates histological analysis of vertebrae of 1 month-old wt (wt 1 mo) and ob/ob mice fed a low fat diet (ob/ob 1 mo LF diet). Figure 2B is a histological analysis of vertebrae of 3 month-old wt and ob/+ mice. Figure 2C is a histological analysis of vertebrae of 6 month-old wt and Agouti yellow mutant mice ( $A^{y/a}$ ). Figure 2D is a histological analysis of vertebrae of 6 month-old wt mice fed a normal diet or a high fat (HF) diet. Underlined numbers indicate a statistically significant difference between experimental and control groups of mice ( $p < 0.05$ ).

**Figures 3A-3H.** Absence of leptin signaling causes an increase in osteoblast function. In Figure 3A, calcein double labeling in 3-month-old wild-type (wt) and ob/ob mice is demonstrated. The distance between the two labels (white arrow) represents the rate of bone formation. In Figures 3B-3F, the rate of bone formation is increased in ob/ob mice (B) and db/db mice (D) 45% and 70%, respectively, compared to wt littermates. This increase occurs in the presence of a normal number of osteoblasts (Figures 3C and 3E), and in spite of the increased number of osteoclasts due to their hypogonadism (Figure 4F). Empty bars, wt mice; black bars, ob/ob mice; grey bars, db/db mice; 3m., 3-month-old animals; 6m., 6-month-old animals. In Figure 4G, increased bone formation rate in fat restricted 1-month-old ob/ob mice and heterozygote ob/+ mice, which are not obese (see body weights Figure 2A and B) is shown. In Figure 3H, wt mice fed a high fat diet, or  $A^{y/a}$  mice, that are overweight (see Figure 2C and D) but not leptin-deficient have a normal rate of bone formation. Asterisks indicate statistically significant differences compared to control mice ( $p < 0.05$ ). Error bars represent SEM.

**Figures 4A-4E.** Normal osteoclasts function in absence of leptin signaling. A comparative analyses of wild-type (wt) and ob/ob mice whose hypogonadism has been corrected by 17  $\beta$ -estradiol treatment (E2) or not corrected (P, placebo) are shown. Figure 4A demonstrates there is correction of the uterus atrophy of the ob/ob mice by the 17  $\beta$ -estradiol treatment. In Figures 4B through 4D there is histological analysis of vertebrae showing that 17  $\beta$ -estradiol treatment leads to a significant increase in bone trabeculae in 17  $\beta$ -estradiol-treated wt and even more in 17  $\beta$ -estradiol-treated ob/ob mice. Grey bars, wild-type mice;

black bars, ob/ob mice; patterned bars, treated mice; solid bars, placebo control mice. Asterisks indicate a statistically significant difference between treated and untreated mice ( $p < 0.05$ ). Error bars represent SEM. In Figure 4E, there is shown normal differentiation and function of ob/ob and db/db osteoclasts *ex-vivo*. Marrow progenitors derived from wt, ob/ob, and db/db mice differentiate equally well in TRAP positive (TRAP+) osteoclasts (upper panel and bottom line). There is also no difference in their ability to form resorption pits on a dentin slice matrix (bottom panel).

**Figures 5A-5F.** Leptin does not signal in osteoblasts. Northern blot analysis of leptin expression in tissues and primary cells (non-mineralizing (NM) osteoblasts, mineralizing (M) osteoblasts and chondrocytes) (upper panel) is demonstrated in Figure 5A. Gapdh expression was used as an internal control for loading (lower panel). Figure 5B shows that *Ob-Rb* (Ob-receptor, type b) transcripts cannot be detected in long bones, calvaria and primary osteoblasts by RT-PCR while this message is detected in hypothalamus. Amplification of *Hprt* was used as an internal control for cDNA quality. Figure 5C is a western blot analysis. Induction by oncostatin-M (OSM) was used as a positive control. In Figure 5D, there is Northern blot analysis of immediate early gene expression (*Tis11* and *c-fos* genes) upon treatment of primary osteoblast cultures with leptin or Oncostatin-M. Gapdh expression was used as an internal control for loading (lower panel). In Figure 5E *ex-vivo* primary osteoblast cultures from wild-type mice maintained in the absence (vehicle) or presence of leptin are shown. No effect on collagen synthesis (upper panel, van Gieson staining) or matrix mineralization (lower panel, von Kossa staining) can be observed. In Figure 5F normal function of db/db osteoblasts in *ex vivo* culture experiments is shown. Collagen synthesis (upper panel, van Gieson staining) and matrix mineralization (lower panel, von Kossa staining) between primary osteoblast cultures derived from wt and db/db mice are demonstrated.

**Figure 6A-6C.** Fat tissue is not required for the appearance of a high bone mass phenotype. In Figure 6A, there is shown histological analysis of vertebrae of 6 month-old wt and A-ZIP/F-1 transgenic mice, that have no fat tissue. Bone volume (B) and bone formation

rate (C) in the transgenic mice are illustrated. Asterisks indicate a statistically significant difference between wt and transgenic mice ( $p < 0.05$ ). Error bars represent SEM.

**Figure 7A-7D.** Leptin action on bone formation is mediated by a hypothalamic relay.

In Figure 7A, there is a histological comparison of vertebrae of 4 month-old ob/ob mice infused centrally (third ventricle) with PBS or leptin and wt mice. Bone volume (B), trabecular volume (C) and the rate of bone formation (D) are demonstrated. Asterisks indicate a statistically significant difference between PBS-infused and leptin-infused mice ( $p < 0.05$ ). Error bars represent SEM.

The drawings and figures are not necessarily to scale and certain features mentioned may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

## **5 DETAILED DESCRIPTION OF THE INVENTION**

Various aspects of the present invention are presented in detail herein.

### **5.1 Leptin and Leptin Receptor Proteins, Polypeptides and Nucleic Acids**

Leptin ("Ob") and leptin receptor ("ObR") proteins and nucleic acids (sense and antisense) can be utilized as part of the therapeutic, diagnostic, prognostic and screening methods of the present invention. For example, Ob and/or ObR proteins, polypeptides and peptide fragments, mutated, truncated or deleted forms of Ob or ObR, including, but not limited to, soluble derivatives such as peptides or polypeptides corresponding to one or more leptin receptor ECDs; truncated leptin receptor polypeptides lacking one or more ECD or TM; and leptin and leptin receptor fusion protein products (such as leptin receptor-Ig fusion proteins, that is, fusions of the leptin receptor or a domain of the leptin receptor, to an IgFc domain) can be utilized.

Sequences of leptin and leptin receptor, including human leptin and leptin receptors, are well known. For a review of leptin receptor proteins, see Friedman and Halaas, 1998, *Nature*, 395:763-770. See, also, U.S. Patent No. 5,972,621. For leptin sequences, including human leptin coding sequences and leptin gene regulatory sequences, see, *e.g.*, Zhang, Y., et al., 1994, *Nature* 372:425-432; de la Brousse et al., 1996, *PNAS* 93:4096-4101; He et al.,

1995, J. Biol. Chem. 270:28887-28891; Hwang et al., 1996, PNAS 93:873-877; and Gong et al., 1996, J. Biol Chem 271:3971-3974.

For example, peptides and polypeptides corresponding to Ob or to one or more domains of the ObR (*e.g.*, ECD, TM or CD), truncated or deleted Ob or ObRs (*e.g.*, ObR in which the TM and/or CD is deleted) as well as fusion proteins in which the full length Ob or ObR, an Ob or ObR peptide or truncated Ob or ObR (*e.g.*, an ObR ECD, TM or CD domain) is fused to a heterologous, unrelated protein are also within the scope of the invention and can be utilized and designed on the basis of such Ob and ObR nucleotide and Ob and ObR amino acid sequences which are known to those of skill in the art. Preferably, leptin polypeptides can bind leptin receptor under standard physiological and/or cell culture conditions.

Likewise, preferably leptin receptor polypeptides can bind leptin under standard physiological and/or cell culture conditions. Thus, at a minimum, leptin receptor polypeptides comprise a leptin amino acid sequence sufficient for leptin receptor binding, that is for leptin/leptin receptor complex formation and likewise, at a minimum, leptin receptor polypeptides comprise a leptin receptor ECD sequence sufficient for leptin binding.

With respect to ObR peptides, polypeptides, fusion peptides and fusion polypeptides comprising all or part of an ObR ECD, such peptides include soluble leptin receptor polypeptides. Preferably, such soluble leptin receptor polypeptides can bind leptin under standard physiological and/or cell culture conditions. Thus, at a minimum, such soluble leptin receptor polypeptides comprise an ObR ECD sequence sufficient for leptin binding.

Fusion proteins include, but are not limited to, IgFc fusions which stabilize the soluble ObR protein or peptide and prolong half-life *in vivo*; or fusions to any amino acid sequence that allows the fusion protein to be anchored to the cell membrane, allowing the ECD to be exhibited on the cell surface; or fusions to an enzyme, fluorescent protein, or luminescent protein which provide a marker or reporter function, useful *e.g.*, in screening and/or diagnostic methods of the invention..

While the Ob and ObR polypeptides and peptides can be chemically synthesized (*e.g.*, see Creighton, 1983, Proteins: Structures and Molecular Principles, W. H. Freeman & Co., N.Y.), large polypeptides derived from Ob and ObR and full length Ob and ObR may advantageously be produced by recombinant DNA technology using techniques well known in the art for expressing nucleic acid containing Ob and ObR gene sequences and/or coding

sequences. Ob and ObR encoding polynucleotides does not refer only to sequences encoding open reading frames, but also to upstream and downstream sequences within the Ob and ObR genes. Such methods also can be used to construct expression vectors containing the Ob and ObR nucleotide sequences. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Press, N.Y., and Ausabel *et al.*, 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y., each of which is incorporated herein by reference in its entirety. Alternatively, RNA capable of encoding Ob and ObR nucleotide sequences may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M. J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

A variety of host-expression vector systems may be utilized to express the Ob and ObR nucleotide sequences of the invention. Where the Ob and ObR peptide or polypeptide is a soluble derivative (*e.g.*, ObR peptides corresponding to the ECD; truncated or deleted ObR in which the TM and/or CD are deleted) the peptide or polypeptide can be recovered from the culture, *ie.*, from the host cell in cases where the ObR peptide or polypeptide is not secreted, and from the culture media in cases where the ObR peptide or polypeptide is secreted by the cells. However, the expression systems also encompass engineered host cells that express Ob and ObR or functional equivalents *in situ*, *i.e.*, anchored in the cell membrane. Purification or enrichment of Ob or ObR from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well known to those skilled in the art. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of Ob and ObR, but to assess biological activity, *e.g.*, in drug screening assays.

The expression systems that may be used for purposes of the invention include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing Ob or ObR nucleotide sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing the nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the

sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing the nucleotide sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3) harboring  
5 recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the Ob or ObR gene product being expressed. For  
10 example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of Ob or ObR protein or for raising antibodies to Ob or ObR protein, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, EMBO J. 2:1791), in which the  
15 Ob or ObR coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion  
20 proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhidrosis virus (AcNPV) is  
25 used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The Ob or ObR gene coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of an Ob or ObR gene coding sequence will result in inactivation of the polyhedrin gene and production of  
30 non-occluded recombinant virus, (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda*

cells in which the inserted gene is expressed. (*E.g.*, see Smith *et al.*, 1983, J. Virol. 46: 584; Smith, U.S. Pat. No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the Ob or ObR nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the Ob or ObR gene product in infected hosts. (*E.g.*, See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted Ob or ObR nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where entire Ob or ObR genes or cDNAs, including their own initiation codons and adjacent sequences, are inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bittner *et al.*, 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such

mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, choroid plexus cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the Ob or ObR sequences may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the Ob or ObR gene products. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of Ob and ObR gene products.

A number of selection systems may be used, including but not limited to, the herpes simplex virus thymidine kinase (Wigler, *et al.*, 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, *et al.*, 1980, Cell 22:817) genes can be employed in tk<sup>-</sup>, hgprt<sup>-</sup> or aprt<sup>-</sup> cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, *et al.*, 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, *et al.*, 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, *et al.*, 1984, Gene 30:147).

The Ob and ObR gene products can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate the transgenic animals.

Any technique known in the art may be used to introduce the Ob or ObR transgene into animals or to "knock-out" or inactivate endogenous Ob or ObR to produce the founder lines of transgenic animals. Such animals can be utilized as part of the screening methods of the invention, and cells and/or tissues from such animals can be obtained for generation of additional compositions (*e.g.*, cell lines, tissue culture systems) that can also be utilized as part of the screening methods of the invention.

Techniques for generation of such animals are well known to those of skill in the art and include, but are not limited to, pronuclear microinjection (Hoppe, P. C. and Wagner, T. E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson *et al.*, 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell 57:717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

With respect to transgenic animals containing a transgenic Ob and/or ObR, such animals can carry an Ob or ObR transgene in all their cells. Alternatively, such animals can carry the transgene or transgenes in some, but not all their cells, *i.e.*, mosaic animals. The transgene may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.* (Lasko, M. *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89: 6232-6236). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous Ob or ObR gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous Ob or ObR gene, respectively. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous Ob or ObR gene in only that cell type, by following, for example, the teaching of Gu *et al.* (Gu, *et al.*, 1994, Science 265: 103-106). The regulatory sequences required for such

a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of Ob and ObR gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the transgene product.

#### **5.1.1 Antibodies to Ob and ObR Proteins**

Antibodies that specifically recognize and bind to one or more epitopes of Ob or ObR, or epitopes of conserved variants of Ob or ObR, or peptide fragments of Ob or ObR can be utilized as part of the methods of the present invention. Such antibodies include, but are not limited to, polyclonal antibodies, monoclonal antibodies (mAbs), human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies and epitope-binding fragments of any of the above.

Such antibodies may be used, for example, as part of the diagnostic or prognostic methods of the invention for diagnosing a bone disease in a mammal by measuring leptin levels in the mammal, *e.g.*, leptin levels in blood serum or cerebrospinal fluid of the mammal. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes, as described below, for the evaluation of the effect of test compounds on expression and/or activity of the Ob or ObR gene product. Additionally, such antibodies can be used in therapeutic and preventative methods of the invention. For example, such antibodies can correspond to leptin receptor agonists or antagonists. Further, such antibodies can be administered to lower leptin levels in the brain, as assayed by leptin levels in cerebrospinal fluid. In addition, such antibodies can be utilized to lower leptin levels by increasing the rate at which leptin is removed from circulation (*e.g.*, can speed leptin breakdown), or can be used

to lower leptin receptor levels, including lowering cells expressing leptin receptor, by increasing the rate at which leptin receptor (and cells expressing leptin receptor) breaks down or is degraded.

For the production of antibodies, various host animals may be immunized by injection with Ob or ObR, an Ob or ObR peptide (*e.g.*, for ObR, one corresponding with a functional domain of the receptor, such as ECD, TM or CD), truncated Ob or ObR polypeptides (*e.g.*, for ObR, in which one or more domains, *e.g.*, the TM or CD, has been deleted), functional equivalents of Ob or ObR or mutants of Ob or ObR. Such host animals may include, but are not limited to, rabbits, mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor *et al.*, 1983, Immunology Today 4:72; Cole *et al.*, 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole *et al.*, 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

Additionally, recombinant antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. A chimeric antibody is a molecule in which different portions are derived from different animal species,

such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. (See, e.g., Cabilly et al., U.S. Patent No. 4,816,567; and Boss et al., U.S. Patent No. 4,816,397, which are incorporated herein by reference in their entirety.) Humanized antibodies are antibody molecules from non-human species having one or more complementarily determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. (See, e.g., Queen, U.S. Patent No. 5,585,089, which is incorporated herein by reference in its entirety.) Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better et al. (1988) *Science* 240:1041-1043; Liu et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu et al. (1987) *J. Immunol.* 139:3521-3526; Sun et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura et al. (1987) *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al. (1988) *J. Natl. Cancer Inst.* 80:1553-1559; Morrison (1985) *Science* 229:1202-1207; Oi et al. (1986) *Bio/Techniques* 4:214; U.S. Patent 5,225,539; Jones et al. (1986) *Nature* 321:552-525; Verhoeyan et al. (1988) *Science* 239:1534; and Beidler et al. (1988) *J. Immunol.* 141:4053-4060.

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Such antibodies can be produced, for example, using transgenic mice which are incapable of expressing endogenous immunoglobulin heavy and light chains genes, but which can express human heavy and light chain genes. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, *Int. Rev. Immunol.* 13:65-93). For a detailed discussion of this technology for producing human antibodies and human

monoclonal antibodies and protocols for producing such antibodies, *see, e.g.*, U.S. Patent 5,625,126; U.S. Patent 5,633,425; U.S. Patent 5,569,825; U.S. Patent 5,661,016; and U.S. Patent 5,545,806. In addition, companies such as Abgenix, Inc. (Fremont, CA), can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al. (1994) *Bio/technology* 12:899-903).

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, *Science* 242:423-426; Huston *et al.*, 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Ward *et al.*, 1989, *Nature* 334:544-546) can be adapted to produce single chain antibodies against Ob and ObR gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed (Huse *et al.*, 1989, *Science*, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to Ob or ObR can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" Ob or ObR, using techniques well known to those skilled in the art. (See, *e.g.*, Greenspan & Bona, 1993, *FASEB J* 7(5):437-444; and Nissinoff, 1991, *J. Immunol.* 147(8):2429-2438). For example, antibodies which bind to the ObR ECD and competitively inhibit the binding of Ob to the ObR can be used to generate anti-idiotypes that "mimic" the ECD and, therefore, bind and neutralize Ob. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize Ob and

treat bone disease characterized by a decreased bone mass relative to a corresponding non-diseased bone.

## 5.2 Diagnosis and Prognosis of Bone Disease and Compound/Patient Monitoring

A variety of methods can be employed for the diagnostic and prognostic evaluation of bone diseases or states, including, but not limited to, osteoporosis, osteopenia, faulty bone formation or resorption, Paget's disease, fractures and broken bones, bone metastasis, osteopetrosis, osteosclerosis and osteochondrosis and for the identification of subjects having a predisposition to such diseases or states.

In particular, bone diseases which can be diagnosed or prognosed in accordance with the present invention include bone diseases characterized by a decreased bone mass relative to that of corresponding non-diseased bone, including, but not limited to osteoporosis, osteopenia and Paget's disease.

Thus, in accordance with this aspect of the present invention, there is a method of diagnosing or prognosing a bone disease in a mammal, such as a human, comprising:

- (a) measuring leptin levels in blood serum of a mammal, *e.g.*, a mammal suspected of exhibiting or being at risk for the bone disease; and
- (b) comparing the level measured in (a) to the leptin level in control blood serum,

so that if the level obtained in (a) is higher than that of the control, the mammal is diagnosed as exhibiting or being at risk for the bone disease, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone.

Alternatively, there is a method of diagnosing or prognosing a bone disease in a mammal, such as a human, comprising:

- (a) measuring leptin levels in cerebrospinal fluid of a mammal, *e.g.*, a mammal suspected of exhibiting or being at risk for the bone disease; and
- (b) comparing the level measured in (a) to the leptin level in control cerebrospinal fluid,

so that if the level obtained in (a) is higher than that of the control, the mammal is diagnosed as exhibiting or being at risk for the bone disease, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone.

5 Further, bone diseases which can be diagnosed or prognosed in accordance with the present invention also include bone diseases characterized by an increased bone mass relative to that of corresponding non-diseased bone, including, but not limited to osteopetrosis, osteosclerosis and osteochondrosis.

Thus, in accordance with this aspect of the present invention, there is a method of diagnosing or prognosing a bone disease in a mammal, such as a human, comprising:

- 10 (a) measuring leptin levels in blood serum of a mammal, *e.g.*, a mammal suspected of exhibiting or being at risk for the bone disease; and
- (b) comparing the level measured in (a) to the leptin level in control blood serum,

15 so that if the level obtained in (a) is lower than that of the control, the mammal is diagnosed as exhibiting or being at risk for the bone disease, wherein the bone disease is characterized by an increased bone mass relative to that of corresponding non-diseased bone.

Alternatively, there is a method of diagnosing or prognosing a bone disease in a mammal, such as a human, comprising:

- 20 (a) measuring leptin levels in cerebrospinal fluid of a mammal, *e.g.*, a mammal suspected of exhibiting or being at risk for the bone disease; and
- (b) comparing the level measured in (a) to the leptin level in control cerebrospinal fluid,

25 so that if the level obtained in (a) is lower than that of the control, the mammal is diagnosed as exhibiting or being at risk for the bone disease, wherein the bone disease is characterized by an increased bone mass relative to that of corresponding non-diseased bone.

30 Additionally, methods are provided for the diagnostic monitoring of patients undergoing clinical evaluation for the treatment of bone disease, and for monitoring the efficacy of compounds in clinical trials.

Thus, yet another aspect of the present invention, there is a method of monitoring efficacy of a compound for treating a bone disease in a mammal, such as a human, comprising:

- 5
- (a) administering the compound to a mammal;
  - (b) measuring leptin levels in blood serum of the mammal; and
  - (c) comparing the level measured in (b) to the leptin level in blood serum of the mammal prior to administering the compound,

thereby monitoring the efficacy of the compound, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Preferred  
10 compounds are ones that increase leptin levels relative to that observed prior to administration.

In accordance with another aspect of the present invention, there is a method of monitoring efficacy of a compound for treating a bone disease in a mammal, such as a human, comprising:

- 15
- (a) administering the compound to a mammal;
  - (b) measuring leptin levels in cerebrospinal fluid of the mammal; and
  - (c) comparing the level measured in (b) to the leptin level in cerebrospinal fluid of the mammal prior to administering the compound,

thereby monitoring the efficacy of the compound, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Preferred  
20 compounds are ones that increase leptin levels relative to that observed prior to administration.

In accordance with yet another aspect of the present invention, there is a method of monitoring efficacy of a compound for treating a bone disease in a mammal, such as a  
25 human, comprising:

- (a) administering the compound to a mammal;
- (b) measuring leptin levels in blood serum of the mammal; and
- (c) comparing the level measured in (b) to the leptin level in blood serum of the mammal prior to administering the compound,

30 thereby monitoring the efficacy of the compound, wherein the bone disease is characterized by a increased bone mass relative to that of corresponding non-diseased bone. Preferred

compounds are ones that decrease leptin levels relative to that observed prior to administration.

In accordance with another aspect of the present invention, there is a method of monitoring efficacy of a compound for treating a bone disease in a mammal, such as a human, comprising:

- (a) administering the compound to a mammal;
- (b) measuring leptin levels in cerebrospinal fluid of the mammal; and
- (c) comparing the level measured in (b) to the leptin level in cerebrospinal fluid of the mammal prior to administering the compound,

thereby monitoring the efficacy of the compound, wherein the bone disease is characterized by a increased bone mass relative to that of corresponding non-diseased bone. Preferred compounds are ones that decrease leptin levels relative to that observed prior to administration.

Methods such as these can also be utilized for monitoring of patients undergoing clinical evaluation for treatment of bone disease. Generally, such methods further include a monitoring of bone mass relative to a corresponding non-diseased bone.

Methods described herein may, for example, utilize reagents such as the Ob and ObR nucleotide sequences described above and known to those of skill in the art (*See, e.g.*, U.S. Patent No. 5,972,621), and Ob and ObR antibodies, as described, in Section 5.1.1. Ob is typically expressed within adipocytes, and lower levels are also found in the stomach and in lymphocytes. ObR is typically expressed in the brain within the hypothalamus. Friedman and Halaas, 1998, *Nature*, 395:763-770. As such, such reagents may be used, for example, for: (1) the detection of the presence of Ob and ObR gene mutations, or the detection of either over- or under-expression of Ob or ObR mRNA relative to the non-bone diseased states, *e.g.*, in a mammal's blood serum or in cerebrospinal fluid; (2) the detection of either an over- or an under-abundance of Ob or ObR gene product relative to the non-bone diseased states, *e.g.*, in a mammal's blood serum or in cerebrospinal fluid; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by Ob or ObR. Alternatively, levels of phosphorylation of Stat3 protein can be measured relative to levels observed in a corresponding control sample or mammal. Stat3 phosphorylation is a biochemical event

which occurs following binding of leptin to the leptin receptor. Devos *et al.*, 1997, JBC, 272:18304-18310.

The methods described herein may be performed in conjunction with, prior to, or subsequent to techniques for measuring bone mass. For example, upon identifying a mammal (e.g., human) exhibiting higher or lower levels of leptin (e.g., in blood serum or cerebrospinal fluid) relative to that of a corresponding control sample, bone mass of the individual can be measured to further clarify whether the mammal exhibits increased or decreased bone mass relative to a corresponding non-diseased bone. If no abnormal bone mass is observed, the mammal can be considered to be at risk for developing disease, while is an abnormal bone mass is observed, the mammal exhibits the bone disease.

Among the techniques well known to those of skill in the art for measuring bone mass are ones that include, but are not limited to, skeletal X-ray, which shows the lucent level of bone (the lower the lucent level, the higher the bone mass); classical bone histology (e.g., bone vlume, number and aspects of trabiculi/trabICATIONS, numbers of osteoblast relative to controls and/or relative to osteoclasts); and dual energy X-ray absorptiometry (DEXA) (Levis and Altman, 1998, Arthritis and Rheumatism, 41:577-587) which measures bone mass and is commonly used in osteoporosis.

The methods described herein may further be used to diagnose individuals at risk for bone disease. Such individuals include, but are not limited to, peri-menopausal women (as used herein, this tem is meant to encompass a time frame from approximately 6 months prior to the onset of menopause to approximately 18 months subsequent to menopause) and patients undergoing treatment with corticosteroids, especially long-term corticosteroid treatment..

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific Ob or ObR nucleotide sequence or Ob or ObR antibody reagent, which may be conveniently used, e.g., in clinical settings, to diagnose patients exhibiting bone diseases.

For the detection of Ob or ObR mutations, any nucleated cell can be used as a starting source for genomic nucleic acid. For the detection of Ob or ObR gene expression or gene products, any cell type or tissue in which the Ob or ObR gene is expressed, such as, for example, choroid plexus cells for the ObR, may be utilized.

Nucleic acid-based detection techniques are described below, in Section 5.2.1.  
Peptide detection techniques are described below, in Section 5.2.2.

### **5.2.1 Detection of Ob and ObR Gene and Transcripts**

5 Mutations within the Ob and ObR gene can be detected by utilizing a number of techniques. Nucleic acid from any nucleated cell can be used as the starting point for such assay techniques, and may be isolated according to standard nucleic acid preparation procedures which are well known to those of skill in the art.

10 DNA may be used in hybridization or amplification assays of biological samples to detect abnormalities involving Ob or ObR gene structure, including point mutations, insertions, deletions and chromosomal rearrangements. Such assays may include, but are not limited to, Southern analyses, single stranded conformational polymorphism analyses (SSCP), and PCR analyses.

15 Such diagnostic methods for the detection of Ob or ObR gene-specific mutations can involve for example, contacting and incubating nucleic acids including recombinant DNA molecules, cloned genes or degenerate variants thereof, obtained from a sample, *e.g.*, derived from a patient sample or other appropriate cellular source, with one or more labeled nucleic acid reagents including recombinant DNA molecules, cloned genes or degenerate variants thereof, under conditions favorable for the specific annealing of these reagents to their  
20 complementary sequences within the Ob or ObR gene, respectively. Preferably, the lengths of these nucleic acid reagents are at least 15 to 30 nucleotides. After incubation, all non-annealed nucleic acids are removed from the nucleic acid:Ob/ObR molecule hybrid. The presence of nucleic acids which have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the nucleic acid from the cell type or tissue of  
25 interest can be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. In this case, after incubation, non-annealed, labeled nucleic acid reagents are easily removed. Detection of the remaining, annealed, labeled Ob or ObR nucleic acid reagents is accomplished using standard techniques well-known to those in the art. The Ob or ObR gene sequences to which the nucleic acid  
30 reagents have annealed can be compared to the annealing pattern expected from a normal Ob or ObR gene sequence in order to determine whether an Ob or ObR gene mutation is present.

Alternative diagnostic methods for the detection of Ob or ObR gene specific nucleic acid molecules, in patient samples or other appropriate cell sources, may involve their amplification, *e.g.*, by PCR (the experimental embodiment set forth in Mullis, K. B., 1987, U.S. Pat. No. 4,683,202), followed by the detection of the amplified molecules using techniques well known to those of skill in the art. The resulting amplified sequences can be compared to those which would be expected if the nucleic acid being amplified contained only normal copies of the Ob or ObR gene in order to determine whether an Ob or ObR gene mutation exists.

Additionally, well-known genotyping techniques can be performed to identify individuals carrying Ob or ObR gene mutations. Such techniques include, for example, the use of restriction fragment length polymorphisms (RFLPs), which involve sequence variations in one of the recognition sites for the specific restriction enzyme used.

Additionally, improved methods for analyzing DNA polymorphisms which can be utilized for the identification of Ob or ObR gene mutations have been described which capitalize on the presence of variable numbers of short, tandemly repeated DNA sequences between the restriction enzyme sites. For example, Weber (U.S. Pat. No. 5,075,217, which is incorporated herein by reference in its entirety) describes a DNA marker based on length polymorphisms in blocks of (dC-dA)<sub>n</sub>-(dG-dT)<sub>n</sub> short tandem repeats. The average separation of (dC-dA)<sub>n</sub>-(dG-dT)<sub>n</sub> blocks is estimated to be 30,000-60,000 bp. Markers which are so closely spaced exhibit a high frequency co-inheritance, and are extremely useful in the identification of genetic mutations, such as, for example, mutations within the Ob or ObR gene, and the diagnosis of diseases and disorders related to Ob or ObR mutations.

Also, Caskey *et al.* (U.S. Pat. No. 5,364,759, which is incorporated herein by reference in its entirety) describe a DNA profiling assay for detecting short tri and tetra nucleotide repeat sequences. The process includes extracting the DNA of interest, such as the Ob or ObR gene, amplifying the extracted DNA, and labeling the repeat sequences to form a genotypic map of the individual's DNA.

The level of Ob or ObR gene expression can also be assayed by detecting and measuring Ob or ObR transcription, respectively. For example, RNA from a cell type or tissue known, or suspected to express the Ob or ObR gene, such as brain, especially choroid plexus cells, may be isolated and tested utilizing hybridization or PCR techniques such as are

described, above. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the Ob or ObR gene. Such analyses may reveal both quantitative and qualitative aspects of the expression pattern of the Ob or ObR gene, including activation or inactivation of Ob or ObR gene expression.

In one embodiment of such a detection scheme, cDNAs are synthesized from the RNAs of interest (e.g., by reverse transcription of the RNA molecule into cDNA). A sequence within the cDNA is then used as the template for a nucleic acid amplification reaction, such as a PCR amplification reaction, or the like. The nucleic acid reagents used as synthesis initiation reagents (e.g., primers) in the reverse transcription and nucleic acid amplification steps of this method are chosen from among Ob and ObR nucleic acid reagents which are well known to those of skill in the art. The preferred lengths of such nucleic acid reagents are at least 9-30 nucleotides. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides. Alternatively, enough amplified product may be made such that the product may be visualized by standard ethidium bromide staining or by utilizing any other suitable nucleic acid staining method.

Additionally, it is possible to perform such Ob and ObR gene expression assays "*in situ*", ie., directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid reagents which are well known to those of skill in the art may be used as probes and/or primers for such *in situ* procedures (See, for example, Nuovo, G. J., 1992, "PCR *In situ* Hybridization: Protocols And Applications", Raven Press, NY).

Alternatively, if a sufficient quantity of the appropriate cells can be obtained, standard Northern analysis can be performed to determine the level of mRNA expression of the Ob and ObR gene.

### **5.2.2 Detection of Ob and ObR Gene Products**

Antibodies directed against wild type or mutant Ob or ObR gene products or conserved variants or peptide fragments thereof, which are discussed, above, in Section 5.1.1,

may also be used as diagnostics and prognostics for bone disease, as described herein. Such diagnostic methods may be used to detect abnormalities in the level of Ob or ObR gene expression, or abnormalities in the structure and/or temporal, tissue, cellular, or subcellular location of Ob or ObR, and may be performed *in vivo* or *in vitro*, such as, for example, on biopsy tissue.

For example, antibodies directed to epitopes of the ObR ECD or Ob can be used *in vivo* to detect the pattern and level of expression of the ObR or Ob in the body. Such antibodies can be labeled, *e.g.*, with a radio-opaque or other appropriate compound and injected into a subject in order to visualize binding to ObR or Ob expressed in the body using methods such as X-rays, CAT-scans, or MRI. Labeled antibody fragments, *e.g.*, the Fab or single chain antibody comprising the smallest portion of the antigen binding region, are preferred for this purpose to promote crossing the blood-brain barrier and permit labeling ObRs expressed in the brain.

Additionally, any Ob or ObR fusion protein or Ob or ObR conjugated protein whose presence can be detected, can be administered. For example, Ob or ObR fusion or conjugated proteins labeled with a radio-opaque or other appropriate compound can be administered and visualized *in vivo*, as discussed, above for labeled antibodies. Further such fusion proteins can be utilized for *in vitro* diagnostic procedures.

Alternatively, immunoassays or fusion protein detection assays, as described above, can be utilized on biopsy and autopsy samples *in vitro* to permit assessment of the expression pattern of Ob or ObR. Such assays are not confined to the use of antibodies that define any particular epitope of Ob or ObR. The use of these labeled antibodies will yield useful information regarding translation and intracellular transport of Ob and ObR to the cell surface, and can identify defects in processing.

The tissue or cell type to be analyzed will generally include those which are known, or suspected, to express the Ob or ObR gene, such as, for example, the hypothalamus and choroid plexus cells for ObR; and adipocytes for Ob. The protein isolation methods employed herein may, for example, be such as those described in Harlow and Lane (Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which is incorporated herein by reference in its entirety. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken

from culture may be a necessary step in the assessment of cells that could be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the Ob or ObR gene.

5 For example, antibodies, or fragments of antibodies, such as those described, above, in Section 5.1.1, useful in the present invention may be used to quantitatively or qualitatively detect the presence of Ob or ObR gene products or conserved variants or peptide fragments thereof. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below, this Section) coupled with light microscopic, flow cytometric, or fluorimetric detection. Such techniques are especially  
10 preferred if such Ob or ObR gene products are expressed on the cell surface.

The antibodies (or fragments thereof) or Ob or ObR fusion or conjugated proteins useful in the present invention may, additionally, be employed histologically, as in immunofluorescence, immunoelectron microscopy or non-immuno assays, for *in situ* detection of Ob and ObR gene products or conserved variants or peptide fragments thereof, or  
15 for Ob binding (in the case of labeled Ob fusion protein).

*In situ* detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody or fusion protein of the present invention. The antibody (or fragment) or fusion protein is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is  
20 possible to determine not only the presence of the Ob or ObR gene product, or conserved variants or peptide fragments, or Ob binding, but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

25 Immunoassays and non-immunoassays for Ob and ObR gene products or conserved variants or peptide fragments thereof will typically comprise incubating a sample, such as a biological fluid (*e.g.*, blood serum or cerebrospinal fluid), a tissue extract, freshly harvested cells, or lysates of cells which have been incubated in cell culture, in the presence of a detectably labeled antibody capable of identifying Ob or ObR gene products or conserved  
30 variants or peptide fragments thereof, and detecting the bound antibody by any of a number of techniques well-known in the art.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled Ob or ObR antibody or Ob or ObR fusion protein. The solid phase support may then be washed with the buffer a second time to remove unbound antibody or fusion protein. The amount of bound label on solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of Ob or ObR antibody or Ob or ObR fusion protein may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

With respect to antibodies, one of the ways in which the Ob or ObR antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA) (Voller, A., "The Enzyme Linked Immunosorbent Assay (ELISA)", 1978, Diagnostic Horizons 2:1-7, Microbiological Associates Quarterly Publication, Walkersville, Md.); Voller, A. *et al.*, 1978, J. Clin. Pathol. 31:507-520; Butler, J. E., 1981, Meth. Enzymol. 73:482-523; Maggio, E. (ed.), 1980, Enzyme Immunoassay, CRC Press, Boca Raton, Fla.,; Ishikawa, E. *et al.*, (eds.), 1981, Enzyme Immunoassay, Kigaku Shoin, Tokyo). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a

chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol  
5 dehydrogenase, alphaglycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by calorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be  
10 accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect Ob or ObR through the use of a radioimmunoassay (RIA) (see, for example,  
15 Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the  
20 fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such  
25 as  $^{152}\text{Eu}$ , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by  
30 detecting the presence of luminescence that arises during the course of a chemical reaction.

Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, therronic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

### 5.3 Screening Assays for Compounds Useful in the Treatment, Diagnosis and Prevention of Bone Disease

The present invention also provides screening methods (*e.g.*, assays) for the identification of compounds which affect bone disease. The invention further encompasses agonists and antagonists of leptin and leptin receptors, including small molecules, large molecules, and antibodies, as well as nucleotide sequences that can be used to inhibit leptin and leptin receptor gene expression (*e.g.*, antisense and ribozyme molecules), and gene or regulatory sequence replacement constructs designed to enhance leptin or leptin receptor gene expression (*e.g.*, expression constructs that place the leptin or leptin receptor gene under the control of a strong promoter system). Such compounds may be used to treat bone diseases.

In particular, cellular and non-cellular assays are described that can be used to identify compounds that interact with leptin and leptin receptors, *e.g.*, modulate the activity of leptin and leptin receptors and/or bind to the leptin receptor. The cell based assays can be used to identify compounds or compositions that affect the signal-transduction activity of leptin and leptin receptors, whether they bind to the leptin receptor or act on intracellular factors involved in the leptin signal transduction pathway. Such cell-based assays of the invention utilize cells, cell lines, or engineered cells or cell lines that express leptin or leptin receptors. The cells can be further engineered to incorporate a reporter molecule linked to the signal transduced by the activated leptin receptor to aid in the identification of compounds that modulate leptin and leptin receptors signaling activity.

The invention also encompasses the use of cell-based assays or cell-lysate assays (*e.g.*, *in vitro* transcription or translation assays) to screen for compounds or compositions

that modulate leptin and leptin receptor gene expression. To this end, constructs containing a reporter sequence linked to a regulatory element of the leptin or leptin receptor genes can be used in engineered cells, or in cell lysate extracts, to screen for compounds that modulate the expression of the reporter gene product at the level of transcription. For example, such assays could be used to identify compounds that modulate the expression or activity of transcription factors involved in leptin and leptin receptor gene expression, or to test the activity of triple helix polynucleotides. Alternatively, engineered cells or translation extracts can be used to screen for compounds (including antisense and ribozyme constructs) that modulate the translation of leptin and leptin receptors mRNA transcripts, and therefore, affect expression of the leptin receptor.

The following assays are designed to identify compounds that interact with (*e.g.*, bind to) Ob or ObR (including, but not limited to, the ECD or CD of ObR), compounds that interact with (*e.g.*, bind to) intracellular proteins that interact with Ob or ObR (including, but not limited to, the TM and CD of ObR), compounds that interfere with the interaction of Ob or ObR with transmembrane or intracellular proteins involved in ObR-mediated signal transduction, and to compounds which modulate the activity of Ob or ObR gene expression or modulate the level of Ob or ObR. Assays may additionally be utilized which identify compounds which bind to Ob or ObR gene regulatory sequences (*e.g.*, promoter sequences) and which may modulate Ob or ObR gene expression. See *e.g.*, Platt, K. A., 1994, J. Biol. Chem. 269:28558-28562 Upon identification, compounds can further be tested for an ability to modulate leptin signalling in vitro or in vivo, and can still further be tested for an ability to modulate bone mass (that is, increase or decrease bone mass) and to treat a bone disease characterized by a decreased or an increased bone mass relative to a corresponding non-diseased bone.

Thus, in accordance with this aspects of the present invention, there is a method for identifying a compound to be tested for an ability to modulate (increase or decrease) bone mass in a mammal, comprising:

- (a) contacting a test compound with a polypeptide; and
- (b) determining whether the test compound binds the polypeptide, so that if the test compound binds the polypeptide, then a compound to be tested for an ability to modulate

bone mass is identified, wherein the polypeptide is selected from the group consisting of a leptin polypeptide and a leptin receptor polypeptide.

Alternatively, there is a method for identifying a compound that modulates (increases or decreases) bone mass in a mammal, comprising:

- 5           (a)     contacting test compounds with a polypeptide;
  - (b)     identifying a test compound that binds the polypeptide; and
  - (c)     administering the test compound in (b) to a non-human mammal, and
- determining whether the test compound modulates bone mass in the mammal relative to that of a corresponding bone in an untreated control non-human mammal, wherein the
- 10   polypeptide is selected from the group consisting of a leptin polypeptide and a leptin receptor polypeptide, so that if the test compound modulates bone mass, then a compound that modulates bone mass in a mammal is identified.

In accordance with this, and other aspects of the present invention, a control non-human mammal, as used herein, is intended to mean a corresponding mammal that has not

15   been administered the test compound

In accordance with yet another aspect of the present invention, there is a method for identifying a compound to be tested for an ability to modulate (increase or decrease) bone mass in a mammal, comprising:

- 20           (a)     contacting a test compound with a leptin polypeptide and a leptin receptor polypeptide for a time sufficient to form leptin/leptin receptor complexes; and
- (b)     measuring leptin/leptin receptor complex level, so that if the level measured differs from that measured in the absence of the test compound, then a compound to be tested for an ability to modulate bone mass is identified.

In accordance with this, and other aspects of the present invention, leptin/leptin

25   receptor complex formation can be measured by, for example, isolating the complex and determining the amount complex formation by various assays well known to those of skill in the art, *e.g.*, Western Blot.

In accordance with another aspect of the present invention, there is a method for identifying a compound to be tested for an ability to decrease bone mass in a mammal,

30   comprising:

(a) contacting a test compound with a cell which expresses a functional leptin receptor; and

(b) determining whether the test compound activates the leptin receptor, wherein if the compound activates the leptin receptor a compound to be tested for an ability to decrease bone mass in a mammal is identified.

In accordance with this, and other aspects of the present invention, a functional leptin receptor is a leptin receptor which is capable of signal transduction following ligand binding to the active site of the receptor. Activation of the leptin receptor, as used herein, is any increase in the activity (*i.e.*, signal transduction) of the leptin receptor.

In accordance with another aspect of the present invention, there is a method for identifying a compound that decreases bone mass in a mammal, comprising:

(a) contacting a test compound with a cell that expresses a functional leptin receptor, and determining whether the test compound activates the leptin receptor;

(b) administering a test compound identified in (a) as activating the leptin receptor to a non-human animal, and determining whether the test compound decreases bone mass of the animal relative to that of a corresponding bone of a control non-human animal, so that if the test compound decreases bone mass, then a compound that decreases bone mass in a mammal is identified.

In accordance with another aspect of the present invention, there is a method for identifying a compound to be tested for an ability to increase bone mass in a mammal, comprising:

(a) contacting a leptin polypeptide and a test compound with a cell that expresses a functional leptin receptor; and

(b) determining whether the test compound lowers activation of the leptin receptor relative to that observed in the absence of the test compound; wherein a test compounds that lowers activation of the leptin receptor is identified as a compound to be tested for an ability to increase bone mass in a mammal.

In accordance with yet another aspect of the present invention, there is a method for identifying a compound that increases bone mass in a mammal, comprising:

(a) contacting a leptin polypeptide and a test compound with a cell that expresses a functional leptin receptor, and determining whether the test compound decreases activation of the leptin receptor;

(b) administering a test compound identified in (a) as decreasing leptin receptor to a non-human animal, and determining whether the test compound increases bone mass of the animal relative to that of a corresponding bone of a control non-human animal, so that if the test compound increases bone mass, then a compound that increases bone mass in a mammal is identified.

In accordance with yet another aspect of the invention, there is a method in which activation of a leptin receptor is determined by measuring levels of phosphorylated Stat3 polypeptide. Stat3 polypeptide, a downstream effector of leptin signaling in its target cells (Tartaglia *et al.*, 1995, *Cell* 83, 1263-1271; Baumann *et al.*, 1996, *Proc Natl Acad Sci USA* 93, 8374-8378; Ghilardi *et al.*, 1996, *Proc Natl Acad Sci USA* 93, 6231-6235; Vaisse *et al.*, 1996, *Nat Genet* 14, 95-97), is phosphorylated following activation of the leptin receptor by leptin.

The compounds which may be screened in accordance with the invention include, but are not limited to, peptides, antibodies and fragments thereof, and other organic compounds (*e.g.*, peptidomimetics) that bind to Ob or ObR and either mimic the activity triggered by the natural ligand (*i.e.*, agonists) or inhibit the activity triggered by the natural ligand (*i.e.*, antagonists); as well as peptides, antibodies or fragments thereof, and other organic compounds that mimic the ECD of the ObR (or a portion thereof) and bind to and "neutralize" natural ligand. Additional compounds which may be screened in accordance with the invention include, but are not limited to, compounds which interact with Ob and prevent the transport of Ob across the blood-brain barrier, thereby preventing Ob from activating the ObR.

Such compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries; (see, *e.g.*, Lam, K. S. *et al.*, 1991, *Nature* 354:82-84; Houghten, R. *et al.*, 1991, *Nature* 354:84-86), and combinatorial chemistry-derived molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; see, *e.g.*, Songyang, Z. *et al.*, 1993, *Cell*

72:767-778), antibodies (including, but not limited to, polyclonal, monoclonal, human, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab')<sub>2</sub> and FAb expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

5           Other compounds which can be screened in accordance with the invention include, but are not limited to, small organic molecules that are able to cross the blood-brain barrier, gain entry into an appropriate cell (*e.g.*, in the choroid plexus or in the hypothalamus) and affect the expression of the Ob or ObR gene or some other gene involved in the ObR signal transduction pathway (*e.g.*, by interacting with the regulatory region or transcription factors involved in gene expression); or such compounds that affect the activity of the ObR (*e.g.*, by  
10           inhibiting or enhancing the enzymatic activity of the CD) or the activity of some other intracellular factor involved in the ObR signal transduction pathway, such as, for example, gp130.

          Computer modeling and searching technologies permit identification of compounds, or the improvement of already identified compounds, that can modulate Ob or ObR  
15           expression or activity. Having identified such a compound or composition, the active sites or regions are identified. Such active sites might typically be ligand binding sites, such as the interaction domains of Ob with ObR itself. The active site can be identified using methods known in the art including, for example, from the amino acid sequences of peptides, from the  
20           nucleotide sequences of nucleic acids, or from study of complexes of the relevant compound or composition with its natural ligand. In the latter case, chemical or X-ray crystallographic methods can be used to find the active site by finding where on the factor the complexed ligand is found. Next, the three dimensional geometric structure of the active site is determined. This can be done by known methods, including X-ray crystallography, which  
25           can determine a complete molecular structure. On the other hand, solid or liquid phase NMR can be used to determine certain intra-molecular distances. Any other experimental method of structure determination can be used to obtain partial or complete geometric structures. The geometric structures may be measured with a complexed ligand, natural or artificial, which may increase the accuracy of the active site structure determined.

30           If an incomplete or insufficiently accurate structure is determined, the methods of computer based numerical modeling can be used to complete the structure or improve its

accuracy. Any recognized modeling method may be used, including parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry. The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

Finally, having determined the structure of the active site, either experimentally, by modeling, or by a combination, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. Such a search seeks compounds having structures that match the determined active site structure and that interact with the groups defining the active site. Such a search can be manual, but is preferably computer assisted. These compounds found from this search are potential Ob or ObR modulating compounds.

Alternatively, these methods can be used to identify improved modulating compounds from an already known modulating compound or ligand. The composition of the known compound can be modified and the structural effects of modification can be determined using the experimental and computer modeling methods described above applied to the new composition. The altered structure is then compared to the active site structure of the compound to determine if an improved fit or interaction results. In this manner, systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain modified modulating compounds or ligands of improved specificity or activity.

Further experimental and computer modeling methods useful to identify modulating compounds based upon identification of the active sites of Ob, ObR, and related transduction and transcription factors will be apparent to those of skill in the art.

Examples of molecular modeling systems are the CHARMM and QUANTA programs (Polygen Corporation, Waltham, Mass.). CHARMM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modelling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific-proteins, such as Rotivinen, *et al.*, 1988, Acta Pharmaceutica Fennica 97:159-166; Ripka, New Scientist 54-57 (Jun. 16, 1988); McKinaly and Rossmann, 1989, Annu. Rev. Pharmacol. Toxicol. 29:111-122; Perry and Davies, OSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989 Proc. R. Soc. Lond. 236:125-140 and 141-162; and, with respect to a model receptor for nucleic acid components, Askew, *et al.*, 1989, J. Am. Chem. Soc. 111:1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc. (Pasadena, Calif.), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Cambridge, Ontario). Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds which are inhibitors or activators.

Compounds identified via assays such as those described herein may be useful, for example, in elaborating the biological function of the Ob or ObR gene product, and for ameliorating bone diseases. Assays for testing the effectiveness of compounds, identified by, for example, techniques such as those described in Section 5.3.1 through 5.3.3, are discussed, below, in Section 5.3.4.

### 5.3.1 In vitro Screening Assays for Compounds that Bind to Ob and ObR

*In vitro* systems may be designed to identify compounds capable of interacting with (*e.g.*, binding to) Ob and ObR (including, but not limited to, the ECD or CD of ObR). Compounds identified may be useful, for example, in modulating the activity of wild type and/or mutant Ob or ObR gene products; may be useful in elaborating the biological function of Ob or ObR; may be utilized in screens for identifying compounds that disrupt normal Ob and ObR interactions; or may in themselves disrupt such interactions.

The principle of the assays used to identify compounds that bind to Ob or ObR involves preparing a reaction mixture of Ob or ObR and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. The Ob or ObR species used can vary depending upon the goal of the screening assay. For example, where agonists of the natural ligand are sought, the full length ObR, or a soluble truncated ObR, *e.g.*, in which the TM and/or CD is deleted from the molecule, a peptide corresponding to the ECD or a fusion protein containing the ObR ECD fused to a protein or polypeptide that affords advantages in the assay system (*e.g.*, labeling, isolation of the resulting complex, etc.) can be utilized. Where compounds that interact with the ObR cytoplasmic domain are sought to be identified, peptides corresponding to the ObR CD and fusion proteins containing the ObR CD can be used. In addition, where compounds which will prevent Ob entry across the blood-brain barrier are sought, Ob, or soluble forms of Ob, can be used.

The screening assays can be conducted in a variety of ways. For example, one method to conduct such an assay would involve anchoring the Ob or ObR protein, polypeptide, peptide or fusion protein or the test substance onto a solid phase and detecting Ob or ObR/test compound complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, the Ob or ObR reactant may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly.

In practice, microtiter plates may conveniently be utilized as the solid phase. The anchored component may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein to be immobilized may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the nonimmobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (*e.g.*, by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously

nonimmobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously nonimmobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; *e.g.*, using a labeled antibody specific for the previously nonimmobilized component (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; *e.g.*, using an immobilized antibody specific for Ob or ObR protein, polypeptide, peptide or fusion protein or the test compound to anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

Alternatively, cell-based assays can be used to identify compounds that interact with Ob or ObR. To this end, cell lines that express Ob or ObR, or cell lines (*e.g.*, COS cells, CHO cells, fibroblasts, etc.) that have been genetically engineered to express Ob or ObR (*e.g.*, by transfection or transduction of Ob or ObR DNA) can be used. Interaction of the test compound with, for example, the ECD of ObR expressed by the host cell can be determined by comparison or competition with native Ob.

### **5.3.2 Assays for Proteins that Interact with Ob and ObR**

Any method suitable for detecting protein-protein interactions may be employed for identifying transmembrane proteins or intracellular proteins that interact with Ob or ObR. Among the traditional methods which may be employed are co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns of cell lysates or proteins obtained from cell lysates and Ob or ObR to identify proteins in the lysate that interact with Ob or ObR. For these assays, the Ob or ObR component used can be full length, a soluble derivative lacking the membrane-anchoring region (*e.g.*, a truncated ObR in which the TM is deleted resulting in a truncated molecule containing the ECD fused to the CD), a peptide corresponding to the CD or a fusion protein containing Ob or the CD of ObR. Once isolated, such an intracellular protein can be identified and can, in turn, be used in conjunction with standard techniques, to identify proteins with which it interacts. For example, at least a portion of the amino acid sequence of an intracellular protein which

interacts with Ob or ObR can be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique. (See, *e.g.*, Creighton, 1983, "Proteins: Structures and Molecular Principles", W.H. Freeman & Co., N.Y., pp.34-49). The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for gene sequences encoding such intracellular proteins. Screening may be accomplished, for example, by standard hybridization or PCR techniques. Techniques for the generation of oligonucleotide mixtures and the screening are well-known. (See, *e.g.*, Ausubel, *supra.*, and PCR Protocols: A Guide to Methods and Applications, 1990, Innis, M. *et al.*, eds. Academic Press, Inc., New York).

Additionally, methods may be employed which result in the simultaneous identification of genes which encode the transmembrane or intracellular proteins interacting with ObR or Ob. These methods include, for example, probing expression libraries in a manner similar to the well known technique of antibody probing of  $\lambda$ gt11 libraries, using labeled Ob or ObR protein, or an Ob or ObR polypeptide, peptide or fusion protein, *e.g.*, an Ob or ObR polypeptide or an Ob or ObR domain fused to a marker (*e.g.*, an enzyme, fluor, luminescent protein, or dye), or an Ig-Fc domain.

One method which detects protein interactions *in vivo*, the two-hybrid system, is described in detail for illustration only and not by way of limitation. One version of this system has been described (Chien *et al.*, 1991, Proc. Natl. Acad. Sci. USA, 88:9578-9582) and is commercially available from Clontech (Palo Alto, Calif.).

Briefly, utilizing such a system, plasmids are constructed that encode two hybrid proteins: one plasmid consists of nucleotides encoding the DNA-binding domain of a transcription activator protein fused to an Ob or ObR nucleotide sequence encoding Ob or ObR, an Ob or ObR polypeptide, peptide or fusion protein, and the other plasmid consists of nucleotides encoding the transcription activator protein's activation domain fused to a cDNA encoding an unknown protein which has been recombined into this plasmid as part of a cDNA library. The DNA-binding domain fusion plasmid and the cDNA library are transformed into a strain of the yeast *Saccharomyces cerevisiae* that contains a reporter gene (*e.g.*, HBS or lacZ) whose regulatory region contains the transcription activator's binding site. Either hybrid protein alone cannot activate transcription of the reporter gene: the DNA-binding domain hybrid cannot because it does not provide activation function and the

activation domain hybrid cannot because it cannot localize to the activator's binding sites. Interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

5           The two-hybrid system or related methodology may be used to screen activation domain libraries for proteins that interact with the "bait" gene product. By way of example, and not by way of limitation, Ob or ObR may be used as the bait gene product. Total genomic or cDNA sequences are fused to the DNA encoding an activation domain. This library and a plasmid encoding a hybrid of a bait Ob or ObR gene product fused to the  
10 DNA-binding domain are cotransformed into a yeast reporter strain, and the resulting transformants are screened for those that express the reporter gene. For example, and not by way of limitation, a bait Ob or ObR gene sequence, such as the open reading frame of Ob or ObR (or a domain of ObR), can be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. These colonies are  
15 purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

A cDNA library of the cell line from which proteins that interact with bait Ob or ObR gene product are to be detected can be made using methods routinely practiced in the art. According to the particular system described herein, for example, the cDNA fragments can be  
20 inserted into a vector such that they are translationally fused to the transcriptional activation domain of GAL4. This library can be co-transformed along with the bait Ob or ObR gene-GAL4 fusion plasmid into a yeast strain which contains a lacZ gene driven by a promoter which contains GAL4 activation sequence. A cDNA encoded protein, fused to GAL4 transcriptional activation domain, that interacts with bait Ob or ObR gene product will  
25 reconstitute an active GAL4 protein and thereby drive expression of the HIS3 gene. Colonies which express HIS3 can be detected by their growth on petri dishes containing semi-solid agar based media lacking histidine. The cDNA can then be purified from these strains, and used to produce and isolate the bait Ob or ObR gene-interacting protein using techniques routinely practiced in the art.

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### 5.3.3 Assays for Compounds that Interfere with Ob and ObR/Intracellular or ObR/Transmembrane Macromolecule Interactions

5       The macromolecules that interact with Ob or ObR are referred to, for purposes of this discussion, as "binding partners". These binding partners are likely to be involved in the ObR signal transduction pathway, and therefore, in the role of Ob or ObR in regulation of bone disorders. Therefore, it is desirable to identify compounds that interfere with or disrupt the interaction of such binding partners with Ob which may be useful in regulating the activity of  
10       the ObR and control bone disorders associated with ObR activity.

      The basic principle of the assay systems used to identify compounds that interfere with the interaction between Ob or ObR and their binding partner or partners involves preparing a reaction mixture containing Ob or ObR protein, polypeptide, peptide or fusion protein as described above, and the binding partner under conditions and for a time sufficient  
15       to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of the Ob or ObR moiety and its binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The  
20       formation of any complexes between the Ob or ObR moiety and the binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of Ob or ObR and the interactive binding partner. Additionally, complex formation within reaction mixtures containing the test compound and normal Ob or ObR protein may also be  
25       compared to complex formation within reaction mixtures containing the test compound and a mutant Ob or ObR. This comparison may be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal Ob or ObRs.

      The assay for compounds that interfere with the interaction of Ob or ObR and binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays  
30       involve anchoring either the Ob or ObR moiety product or the binding partner onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach,

the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction by competition can be identified by conducting the reaction in the presence of the test substance; *i.e.*, by adding the test substance to the reaction mixture prior to or simultaneously with the Ob or ObR moiety and interactive binding partner. Alternatively, test compounds that disrupt preformed complexes, *e.g.* compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, either the Ob or ObR moiety or the interactive binding partner, is anchored onto a solid surface, while the non-anchored species is labeled, either directly or indirectly. In practice, microtiter plates are conveniently utilized. The anchored species may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the Ob or ObR gene product or binding partner and drying. Alternatively, an immobilized antibody specific for the species to be anchored may be used to anchor the species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (*e.g.*, by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; *e.g.*, using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; *e.g.*, using an immobilized antibody specific for one of the binding

components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt preformed complexes can be identified.

5 In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the Ob or ObR moiety and the interactive binding partner is prepared in which either Ob or ObR or its binding partners is labeled, but the signal generated by the label is quenched due to formation of the complex (see, *e.g.*, U.S. Pat. No. 4,109,496 by Rubenstein which utilizes this approach for immunoassays). The addition of a  
10 test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances which disrupt Ob or ObR/intracellular binding partner interaction can be identified.

In a particular embodiment, an Ob or ObR fusion can be prepared for immobilization.  
15 For example, the Ob or ObR or a peptide fragment, *e.g.*, corresponding to the ObR CD, can be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive binding partner can be purified and used to raise a monoclonal antibody, using methods routinely practiced in the art. This antibody can be labeled with the  
20 radioactive isotope <sup>125</sup>I, for example, by methods routinely practiced in the art. In a heterogeneous assay, *e.g.*, the GST-ObR fusion protein can be anchored to glutathione-agarose beads. The interactive binding partner can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled  
25 monoclonal antibody can be added to the system and allowed to bind to the complexed components. The interaction between the Ob or ObR gene product and the interactive binding partner can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

30 Alternatively, the GST-Ob/ObR fusion protein and the interactive binding partner can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test

compound can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the Ob or ObR/binding partner interaction can be detected by adding the labeled antibody and measuring the radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domains of Ob or ObR and/or the interactive or binding partner (in cases where the binding partner is a protein), in place of one or both of the full length proteins. Any number of methods routinely practiced in the art can be used to identify and isolate the binding sites. These methods include, but are not limited to, mutagenesis of the gene encoding one of the proteins and screening for disruption of binding in a co-immunoprecipitation assay. Compensating mutations in the gene encoding the second species in the complex can then be selected. Sequence analysis of the genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described above, and allowed to interact with and bind to its labeled binding partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a short, labeled peptide comprising the binding domain may remain associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene coding for the intracellular binding partner is obtained, short gene segments can be engineered to express peptide fragments of the protein, which can then be tested for binding activity and purified or synthesized.

For example, and not by way of limitation, an Ob or ObR gene product can be anchored to a solid material as described, above, by making a GST-Ob or -ObR fusion protein and allowing it to bind to glutathione agarose beads. The interactive binding partner can be labeled with a radioactive isotope, such as  $^{35}\text{S}$ , and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-Ob or -ObR fusion protein and allowed to bind. After washing away unbound peptides, labeled bound material, representing the intracellular binding partner binding domain, can be eluted, purified, and analyzed for amino acid sequence by well-known methods. Peptides so

identified can be produced synthetically or fused to appropriate facilitative proteins using recombinant DNA technology.

#### 5.3.4 Assays for Identification of Compounds that Ameliorate Bone Disease

Compounds, including, but not limited to, compounds identified via assay techniques such as those described, above, in Sections 5.3.1 through 5.3.3, can be tested for the ability to treat bone disease and ameliorate bone disease symptoms. The assays described above can identify compounds which affect Ob or ObR activity (*e.g.*, leptin receptor agonists or antagonists), and compounds that bind to the natural ligand of the ObR and neutralize ligand activity; or compounds that affect Ob or ObR gene activity (by affecting Ob or ObR gene expression, including molecules, *e.g.*, proteins or small organic molecules, that affect or interfere with splicing events so that expression of the full length or the truncated form of the Ob or ObR can be modulated). However, it should be noted that the assays described can also identify compounds that modulate Ob or ObR signal transduction (*e.g.*, compounds which affect downstream signaling events, such as inhibitors or enhancers of tyrosine kinase or phosphatase activities which participate in transducing the signal activated by Ob binding to the ObR). Alternatively, the assays described can also identify compounds which modulate the entry of Ob through the blood-brain barrier. The identification and use of such compounds which affect another step in the Ob or ObR signal transduction pathway in which the Ob or ObR gene and/or gene product is involved and, by affecting this same pathway may modulate the effect of Ob or ObR on the development of bone disorders are within the scope of the invention. Such compounds can be used as part of a therapeutic method for the treatment of bone disease.

Cell-based systems can be used to identify compounds which may act to ameliorate bone disease. Such cell systems can include, for example, recombinant or non-recombinant cells, such as cell lines, which express the Ob or ObR gene, *e.g.*, NIH3T3L1 cell lines. Further, for example, for ObR, choroid plexus cells, hypothalamus cells, or cell lines derived from choroid plexus or hypothalamus can be used. In addition, expression host cells (*e.g.*, COS cells, CHO cells, fibroblasts) genetically engineered to express a functional Ob or ObR and to respond to activation by the natural Ob ligand, *e.g.*, as measured by a chemical or

phenotypic change, induction of another host cell gene, change in ion flux (*e.g.*,  $\text{Ca}^{++}$ ), tyrosine phosphorylation of host cell proteins, etc., can be used as an end point in the assay.

In utilizing such cell systems, cells may be exposed to a compound suspected of exhibiting an ability to ameliorate bone disorders, at a sufficient concentration and for a time sufficient to elicit such an amelioration of bone disorders in the exposed cells. After exposure, the cells can be assayed to measure alterations in the expression of the Ob or ObR gene, *e.g.*, by assaying cell lysates for Ob or ObR mRNA transcripts (*e.g.*, by Northern analysis) or for Ob or ObR protein expressed in the cell; compounds which regulate or modulate expression of the Ob or ObR gene are good candidates as therapeutics.

Alternatively, the cells are examined to determine whether one or more bone disorder-like cellular phenotypes has been altered to resemble a more normal or more wild type, non-bone disorder phenotype, or a phenotype more likely to produce a lower incidence or severity of disorder symptoms. Still further, the expression and/or activity of components of the signal transduction pathway of which ObR is a part, or the activity of the ObR signal transduction pathway itself can be assayed.

For example, after exposure, the cell lysates can be assayed for the presence of tyrosine phosphorylation of host cell proteins, as compared to lysates derived from unexposed control cells. The ability of a test compound to inhibit tyrosine phosphorylation of host cell proteins in these assay systems indicates that the test compound inhibits signal transduction initiated by ObR activation. The cell lysates can be readily assayed using a Western blot format; *i.e.*, the host cell proteins are resolved by gel electrophoresis, transferred and probed using a anti-phosphotyrosine detection antibody (*e.g.*, an anti-phosphotyrosine antibody labeled with a signal generating compound, such as radiolabel, fluor, enzyme, etc.) (See, *e.g.*, Glenney *et al.*, 1988, J. Immunol. Methods 109:277-285; Frackelton *et al.*, 1983, Mol. Cell. Biol. 3:1343-1352). Alternatively, an ELISA format could be used in which a particular host cell protein involved in the ObR signal transduction pathway is immobilized using an anchoring antibody specific for the target host cell protein, and the presence or absence of phosphotyrosine on the immobilized host cell protein is detected using a labeled anti-phosphotyrosine antibody. (See, King *et al.*, 1993, Life Sciences 53:1465-1472). In yet another approach, ion flux, such as calcium ion flux, can be measured as an end point for ObR stimulated signal transduction.

In addition, animal-based bone disorder systems, which may include, for example, ob, db and ob/db mice, may be used to identify compounds capable of ameliorating bone disorder-like symptoms. Such animal models may be used as test substrates for the identification of drugs, pharmaceuticals, therapies and interventions which may be effective in treating such disorders. For example, animal models may be exposed to a compound suspected of exhibiting an ability to ameliorate bone disorder symptoms, at a sufficient concentration and for a time sufficient to elicit such an amelioration of bone disorder symptoms in the exposed animals. The response of the animals to the exposure may be monitored by assessing the reversal of disorders associated with bone disorders such as osteoporosis. With regard to intervention, any treatments which reverse any aspect of bone disorder-like symptoms should be considered as candidates for human bone disorder therapeutic intervention. Dosages of test agents may be determined by deriving dose-response curves, as discussed below.

#### 5.4 Compounds that Modulate Ob or ObR Expression or Activity

Compounds that interact with (*e.g.*, bind to) Ob or ObR (including, but not limited to, the ECD or CD of ObR), compounds that interact with (*e.g.*, bind to) intracellular proteins that interact with Ob or ObR (including, but not limited to, the TM and CD of ObR), compounds that interfere with the interaction of Ob or ObR with transmembrane or intracellular proteins involved in ObR-mediated signal transduction, and compounds which modulate the activity of Ob or ObR gene expression or modulate the level of Ob or ObR are capable of modulating levels of bone mass. More specifically, compounds which decrease the levels of Ob or ObR, inhibit the transport of Ob across the blood-brain barrier or inhibit binding of Ob to the ObR would cause an increase in bone mass.

Examples of such compounds are leptin and leptin receptor agonists and antagonists. Leptin receptor antagonist, as used herein, refers to a factor which neutralizes or impedes or otherwise reduces the action or effect of a leptin receptor. Such antagonists can include compounds that bind leptin or that bind leptin receptor. Such antagonists can also include compounds that neutralize, impede or otherwise reduce leptin receptor output, that is, intracellular steps in the leptin signaling pathway following binding of leptin to the leptin receptor, *i.e.*, downstream events that affect leptin/leptin receptor signaling, that do not occur

at the receptor/ligand interaction level. Leptin receptor antagonists may include, but are not limited to proteins, antibodies, small organic molecules or carbohydrates, such as, for example, acetylphenol compounds, antibodies which specifically bind leptin, antibodies which specifically bind leptin receptor, and compounds that comprise soluble leptin receptor polypeptide sequences.

For example, leptin antagonists also include agents, or drugs, which decrease, inhibit, block, abrogate or interfere with binding of leptin to its receptors or extracellular domains thereof; agents which decrease, inhibit, block, abrogate or interfere with leptin production or activation; agents which are antagonists of signals that drive leptin production or synthesis, and agents which prohibit leptin from reaching its receptor, *e.g.*, prohibit leptin from crossing the blood-brain barrier. Such an agent can be any organic molecule that inhibits or prevents the interaction of leptin with its receptor, or leptin production. *See*, U.S. Patent No. 5,866,547.

Leptin receptor agonist, as used herein, refers to a factor which activates, induces or otherwise increases the action or effect of a leptin receptor. Such agonists can include compounds that bind leptin or that bind leptin receptor. Such antagonists can also include compounds that activate, induce or otherwise increase leptin receptor output, that is, intracellular steps in the leptin signaling pathway following binding of leptin to the leptin receptor, *i.e.*, downstream events that affect leptin/leptin receptor signaling, that do not occur at the receptor/ligand interaction level. Leptin receptor agonists may include, but are not limited to proteins, antibodies, small organic molecules or carbohydrates, such as, for example, leptin, leptin analogs, and antibodies which specifically bind and activate leptin. Leptin antagonists include, but are not limited to, anti-leptin antibodies, receptor molecules and derivatives which bind specifically to leptin and prevent leptin from binding to its cognate receptor.

Additional Ob binding proteins include, but are not limited to, inter-alpha-trypsin inhibitor heavy chain-related protein (IHRP); alpha 2-macroglobulin; and OB-BP1 which specifically bind Ob and is thus capable of preventing Ob from binding to the ObR. The specific Ob binding protein further enables modulation of free Ob levels, immobilization and assay of bound/free leptin. *See*, U.S. Patent No. 5,919,902; Birkenmeier *et al.*, 1998, Eur. J.

Endocrin., 139:224-230; Patel *et al.*, 1999, J. Biol. Chem., 32:22729-22738. Additional Ob binding proteins, such as apolipoproteins, are disclosed in U.S. Patent No. 5,830,450.

Examples of ObR antagonists are acetylphenols, which are known to be useful as antiobesity and antidiabetic compounds. Since acetylphenols are antagonists of the ObR, they prevent binding of Ob to the ObR. Thus, in view of the teachings of the present invention, the compounds would effectively cause an increase in bone mass. For specific structures of acetylphenols which can be used as ObR antagonists, see U.S. Patent No. 5,859,051.

Additional antagonists and agonists of the ObR, and other compounds that modulate ObR gene expression or ObR activity that can be used for diagnosis, drug screening, clinical trial monitoring, and/or the treatment of bone disorders can be found in U.S. Patent Nos. 5,972,621, 5,874,535, and 5,912,123.

#### **5.5. Methods for the Treatment or Prevention of Bone Disease**

Bone diseases which can be treated and/or prevented in accordance with the present invention include bone diseases characterized by a decreased bone mass relative to that of corresponding non-diseased bone, including, but not limited to osteoporosis, osteopenia and Paget's disease. Bone diseases which can be treated and/or prevented in accordance with the present invention also include bone diseases characterized by an increased bone mass relative to that of corresponding non-diseased bone, including, but not limited to osteopetrosis, osteosclerosis and osteochondrosis.

In one aspect of the invention is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound that lowers leptin level in blood serum, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin synthesis or increase leptin breakdown. Among such compounds are antisense, ribozyme or triple helix sequences of a leptin-encoding polypeptide.

In accordance with another aspect of the present invention, there is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound that lowers leptin level in cerebrospinal fluid,

wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin synthesis or increase leptin breakdown, and compounds that bind leptin in blood.

5 Particular embodiments of the methods of the invention include, for example, a method of treating a bone disease comprising: administering to a mammal in need of said treatment a therapeutically effective amount of a compound, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone, and wherein the compound is selected from the group consisting of compounds which bind  
10 leptin in blood, including, but not limited to such compounds as an antibody which specifically binds leptin, a soluble leptin receptor polypeptide, an inter-alpha-trypsin inhibitor heavy chain related protein and an alpha 2-macroglobulin protein.

In accordance with another aspect of the present invention, there is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a  
15 therapeutically effective amount of a compound that lowers the level of phosphorylated Stat3 polypeptide, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin synthesis or increase leptin breakdown, compounds that bind leptin in blood, and leptin receptor  
20 antagonist compounds, such as acetylphenol compounds, antibodies which specifically bind leptin, antibodies which specifically bind leptin receptor, and compounds that comprise soluble leptin receptor polypeptide sequences.

In accordance with another aspect of the present invention, there is a method of treating a bone disease comprising: administering to a mammal in need of said treatment a  
25 therapeutically effective amount of a compound that lowers leptin receptor levels in hypothalamus, wherein the bone disease is characterized by a decreased bone mass relative to that of corresponding non-diseased bone. Specific embodiments of some of these compounds and methods include, but are not limited to ones that inhibit or lower leptin receptor synthesis or increase leptin receptor breakdown. Among such compounds are antisense, ribozyme or  
30 triple helix sequences of a leptin receptor-encoding polypeptide.

A compound that lowers leptin levels in blood serum or in cerebrospinal fluid is one that lowers leptin levels in the following assay: contacting the compound with a cell from a leptin expressing cell line, preferably a NIH3T3L1 cell line, and determining whether leptin expression and/or synthesis is lowered relative to the level exhibited by the cell line in the absence of the compound. Standard assays such as Northern Blot can be used to determine levels of leptin expression and Western Blot can be used to determine levels of leptin synthesis. An alternate assay comprises comparing the level of leptin in a mammal being treated for a bone disease before and after administration of the compound, such that, if the level of leptin decreases, the compound is one that lowers leptin levels. Likewise, a compound that increases leptin levels in blood serum or in cerebrospinal fluid is one that increases leptin levels via such assays.

A compound that lowers the level of phosphorylated Stat3 polypeptide, a downstream effector of leptin signaling in its target cells (Tartaglia *et al.*, 1995, *Cell* 83, 1263-1271; Baumann *et al.*, 1996, *Proc Natl Acad Sci USA* 93, 8374-8378; Ghilardi *et al.*, 1996, *Proc Natl Acad Sci USA* 93, 6231-6235; Vaisse *et al.*, 1996, *Nat Genet* 14, 95-97), is one that lowers the level of phosphorylated Stat3 in the following assay: contacting a leptin polypeptide and the compound with a cell that expresses a functional leptin receptor and determining the level of phosphorylated Stat3 polypeptide in the cell. To determine the level of phosphorylation of Stat3 polypeptide, the cells can, for example, be lysed and an appropriate analysis (*e.g.*, Western Blot) can be performed. If the level of phosphorylated Stat3 decreases relative to the level exhibited by the cell line in the absence of the compound, the compound is one that lowers the level of phosphorylated Stat3. Likewise, a compound that increases the level of phosphorylated Stat3 polypeptide in blood serum or in cerebrospinal fluid is one that increases leptin levels via such assays.

A compound is said to be administered in a "therapeutically effective amount" if the amount administered results in a desired change in the physiology of a recipient mammal, *e.g.*, results in an increase or decrease in bone mass relative to that of a corresponding bone in the diseased state; that is, results in treatment, *i.e.*, modulates bone mass to more closely resemble that of corresponding non-diseased bone (that is a corresponding bone of the same type, *e.g.*, long, vertebral, *etc.*) in a non-diseased state. With respect to these methods, a corresponding non-diseased bone refers to a bone of the same type as the bone being treated.

(e.g., a corresponding vertebral or long bone), and bone mass is measured using standard techniques well known to those of skill in the art and described above, and include, for example, X-ray, DEXA and classical histological assessments and measurements of bone mass.

5 Among the compounds that can be utilized as part of the methods presented herein are those described, for example, in the sections and taught presented herein, as well as compounds identified via techniques such as those described in the sections and teaching presented herein.

10 Particular techniques and methods that can be utilized as part of the therapeutic and preventative methods of the invention are presented in detail below.

#### **5.5.1. Inhibition of Ob or ObR Expression, Levels or Activity to Treat Bone Disease by Increasing Bone Mass**

15 Any method which neutralizes, slows or inhibits Ob or ObR expression (either transcription or translation), levels, or activity can be used to treat or prevent a bone disease characterized by a decrease in bone mass relative to a corresponding non-diseased bone by effectuating an increase in bone mass. Such approaches can be used to treat or prevent bone diseases such as osteoporosis, osteopenia, faulty bone formation or resorption, Paget's  
20 disease, and bone metastasis. Such methods can be utilized to treat states involving bone fractures and broken bones.

For example, the administration of compounds such as soluble peptides, proteins, fusion proteins, or antibodies (including anti-idiotypic antibodies) that bind to and "neutralize" circulating Ob, the natural ligand for the ObR, can be used to effectuate an  
25 increase in bone mass. Similarly, such compounds as soluble peptides, proteins, fusion proteins, or antibodies (including anti-idiotypic antibodies) can be used to effectuate an increase in bone mass. To this end, peptides corresponding to the ECD of ObR, soluble deletion mutants of ObR, or either of these ObR domains or mutants fused to another polypeptide (e.g., an IgFc polypeptide) can be utilized. Alternatively, anti-idiotypic  
30 antibodies or Fab fragments of antiidiotypic antibodies that mimic the ObR ECD and neutralize Ob can be used. Alternatively, compounds that inhibit ObR homodimerization such that leptin's affinity for the leptin receptor is decreased, also can be used. Devos *et al.*,

1997, JBC, 272:18304-18310. For treatment, such ObR peptides, proteins, fusion proteins, anti-idiotypic antibodies or Fabs are administered to a subject in need of treatment at therapeutically effective levels. For prevention, such ObR peptides, proteins, fusion proteins, anti-idiotypic antibodies or Fabs are administered to a subject at risk for a bone disease, for a time and concentration sufficient to prevent the bone disease.

In an alternative embodiment for neutralizing circulating Ob, cells that are genetically engineered to express such soluble or secreted forms of ObR may be administered to a patient, whereupon they will serve as "bioreactors" *in vivo* to provide a continuous supply of the Ob neutralizing protein. Such cells may be obtained from the patient or an MHC compatible donor and can include, but are not limited to, fibroblasts, blood cells (*e.g.*, lymphocytes), adipocytes, muscle cells, endothelial cells etc. The cells are genetically engineered *in vitro* using recombinant DNA techniques to introduce the coding sequence for the ObR ECD, or for ObR-Ig fusion protein into the cells, *e.g.*, by transduction (using viral vectors, and preferably vectors that integrate the transgene into the cell genome) or transfection procedures, including, but not limited to, the use of plasmids, cosmids, YACs, electroporation, liposomes, etc. The ObR coding sequence can be placed under the control of a strong constitutive or inducible promoter or promoter/enhancer to achieve expression and secretion of the ObR peptide or fusion protein. The engineered cells which express and secrete the desired ObR product can be introduced into the patient systemically, *e.g.*, in the circulation, intraperitoneally, at the choroid plexus or hypothalamus. Alternatively, the cells can be incorporated into a matrix and implanted in the body, *e.g.*, genetically engineered fibroblasts can be implanted as part of a skin graft; genetically engineered endothelial cells can be implanted as part of a vascular graft. (See, for example, Anderson *et al.* U.S. Pat. No. 5,399,349; and Mulligan & Wilson, U.S. Pat. No. 5,460,959 each of which is incorporated by reference herein in its entirety).

When the cells to be administered are non-autologous cells, they can be administered using well known techniques which prevent the development of a host immune response against the introduced cells. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

In an alternate embodiment, bone disease therapy can be designed to reduce the level of endogenous Ob or ObR gene expression, *e.g.*, using antisense or ribozyme approaches to inhibit or prevent translation of Ob or ObR mRNA transcripts; triple helix approaches to inhibit transcription of the Ob or ObR gene; or targeted homologous recombination to inactivate or "knock out" the Ob or ObR gene or its endogenous promoter. Because the ObR gene is expressed in the brain, including the choroid plexus and hypothalamus, delivery techniques should be preferably designed to cross the blood-brain barrier (see PCT WO89/10134, which is incorporated by reference herein in its entirety). Alternatively, the antisense, ribozyme or DNA constructs described herein could be administered directly to the site containing the target cells; *e.g.*, the choroid plexus, hypothalamus, adipose tissue, etc.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to Ob or ObR mRNA. The antisense oligonucleotides will bind to the complementary Ob or ObR mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

The skilled artisan recognizes that modifications of gene expression can be obtained by designing antisense molecules to the control regions of the leptin or leptin receptor genes, *i.e.* promoters, enhancers, and introns, as well as to the coding regions of these genes. Such sequences are referred to herein as leptin-encoding polynucleotides or leptin receptor-encoding polynucleotides, respectively.

Oligonucleotides derived from the transcription initiation site, *e.g.* between -10 and +10 regions of the leader sequence, are preferred. Oligonucleotides that are complementary to the 5' end of the message, *e.g.*, the 5' untranslated sequence up to and including the AUG

initiation codon, generally work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently shown to be effective at inhibiting translation of mRNAs as well. See generally, Wagner, R., 1994, Nature 372:333-335. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5'-, 3'- or coding region of Ob or ObR mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.*, 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre *et al.*, 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO88/09810, published Dec. 15, 1988) or the blood-brain barrier (see, *e.g.*, PCT Publication No. WO89/10134, published Apr. 25, 1988), hybridization-triggered cleavage agents. (See, *e.g.*, Krol *et al.*, 1988, BioTechniques 6:958-976) or intercalating

agents. (See, *e.g.*, Zon, 1988, Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an  $\alpha$ -anomeric oligonucleotide. An  $\alpha$ -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gautier *et al.*, 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue *et al.*, 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue *et al.*, 1987, FEBS Lett. 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al.* (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

While antisense nucleotides complementary to the Ob or ObR coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred. For example, antisense oligonucleotides to the ObR coding region having the following sequences can be utilized in accordance with the invention:

- 10 AI
- a) 5'-CATCTTACTTCAGAGAA-3'
- b) 5'-CATCTTACTTCAGAGAAGTACAC-3'
- c) 5'-CATCTTACTTCAGAGAAGTACACCCATAA-3'
- d) 5'-CATCTTACTTCAGAGAAGTACACCCATAATCCTCT-3'
- 15 e) 5'-AATCATCTTACTTCAGAGAAGTACACCCATAATCC-3'
- f) 5'-CTTACTTCAGAGAAGTACACCCATAATCC-3'
- g) 5'-TCAGAGAAGTACACCCATAATCC-3'
- h) 5'-AAGTACACCCATAATCC-3'

The antisense molecules should be delivered to cells which express the Ob or ObR *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

25 A preferred approach for achieving intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs that will form

30 complementary base pairs with the endogenous Ob or ObR transcripts and thereby prevent translation of the Ob or ObR mRNA, respectively. For example, a vector can be introduced *in*

*vivo* such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296:39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site; *e.g.*, the choroid plexus or hypothalamus. Alternatively, viral vectors can be used which selectively infect the desired tissue; (*e.g.*, for brain, herpesvirus vectors may be used), in which case administration may be accomplished by another route (*e.g.*, systemically).

Ribozyme molecules-designed to catalytically cleave Ob or ObR mRNA transcripts can also be used to prevent translation of Ob or ObR mRNA and expression of Ob or ObR. (See, *e.g.*, PCT International Publication WO90/11364, published Oct. 4, 1990; Sarver *et al.*, 1990, Science 247:1222-1225). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy Ob or ObR mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, 1988, Nature, 334:585-591. There are hundreds of potential hammerhead ribozyme cleavage sites within the nucleotide sequence of human Ob and ObR cDNA. See, *e.g.*, U.S. Patent No. 5,972,621. Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the Ob or ObR

mRNA; *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

For example, hammerhead ribozymes directed to ObR mRNA having the following sequences can be utilized in accordance with the invention:

5 a) 5'-ACAGAAUUUUUGACAAAUCAAAGCAGANNNUCUGAGNAGUCCUACUUC  
AGAGAA-3';

b) 5'-GGCCCGGGCAGCCUGCCCAAAGCCGGNNNNCCGGAGNAGUCGCCAGACCG  
GCUCGUG-3';

10 c) 5'-UGGCAUGCAAGACA AAGCAGGNNNNCCUGAGNAGUCCUAAAUCUCCAAG  
GAGUAA-3';

d) 5'-UAUAUGACAAAGCUGUNNNNACAGAGNAGUCCUUGUGUGGUAAAGACACG  
-3';

15 e) 5'-AGCACCAAUUGAAUUGAUUGGCCAAAGCGGGNNNNCCCGAGNAGUCAACCG  
UAACAGUAUGU-3';

f) 5'-UGAAAUUGUUUCAGGCUCCAAAGCCGGNNNNCCGGAGNAGUCAAGAAGAG  
GACCACAUGUCACUGAUGC-3';

20 g) 5'-GGUUUCUUCAGUGAAAUACACAAAGCAGCNNNNGCUGAGNAGUCAGUUA  
GGUCACACAUC-3';

h) 5'-ACCCAUAUAACACAAAGCUGANNNUCAGAGNAGUCAUCUGAAGGUUUC  
UUC -3'.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, *et al.*, 1984, Science, 224:574-578; Zaug and Cech, 1986, Science, 231:470-475; Zaug, *et al.*, 1986, Nature, 324:429-433; published International patent-application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in Ob and ObR.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.* for improved stability, targeting, etc.) and should be delivered to cells which express Ob and ObR *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous Ob or ObR messages and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Similarly, leptin or leptin receptor inhibition can be achieved by using "triple helix" base-pairing methodology. Triple helix pairing compromises the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Techniques for utilizing triple helix technology are well known to those of skill in the art. *See generally* Helene (1991) *Anticancer Drug Des.* 6(6):569-84; Helene (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher (1992) *Bioassays* 14(12):807-15.

Endogenous Ob or ObR gene expression can also be reduced by inactivating or "knocking out" the Ob or ObR gene or its promoter using targeted homologous recombination. (*E.g.*, see Smithies *et al.*, 1985, *Nature* 317:230-234; Thomas & Capecchi, 1987, *Cell* 51:503-512; Thompson *et al.*, 1989 *Cell* 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional Ob or ObR (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous Ob or ObR gene (either the coding regions or regulatory regions) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express Ob or ObR *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the Ob or ObR gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive ObR (*e.g.*, see Thomas & Capecchi 1987 and Thompson 1989, *supra*). However, this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors, *e.g.*, herpes virus vectors for delivery to brain tissue; *e.g.*, the hypothalamus, choroid plexus, or adipose tissue.

Alternatively, endogenous Ob or ObR gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the Ob or ObR gene (*i.e.*, promoters and/or enhancers) to form triple helical structures that prevent transcription of the Ob or ObR gene in target cells in the body. (See generally, Helene, C. 1991, *Anticancer Drug Des.*, 6(6):569-84; Helene, C., *et al.*, 1992, *Ann. N.Y. Acad. Sci.*, 660:27-36; and Maher, L. J., 1992, *Bioassays* 14(12):807-15).

In yet another embodiment of the invention, the activity of Ob or ObR can be reduced using a "dominant negative" approach to effectuate an increase in bone mass. To this end, constructs which encode defective Ob or ObRs can be used in gene therapy approaches to diminish the activity of the Ob or ObR in appropriate target cells. For example, nucleotide sequences that direct host cell expression of ObRs in which the CD or a portion of the CD is deleted or mutated can be introduced into cells in the choroid plexus or hypothalamus (either by *in vivo* or *ex vivo* gene therapy methods described above). Alternatively, targeted homologous recombination can be utilized to introduce such deletions or mutations into the subject's endogenous ObR gene in the hypothalamus or choroid plexus. The engineered cells will express non-functional receptors (*i.e.*, an anchored receptor that is capable of binding its natural ligand, but incapable of signal transduction). Such engineered cells present in the choroid plexus or hypothalamus should demonstrate a diminished response to the endogenous Ob ligand, resulting in an increase in bone mass.

An additional embodiment of the present invention is a method to decrease leptin levels by increasing breakdown of leptin protein, *i.e.*, by binding of an antibody such that the leptin protein is targeted for removal. An alternative embodiment of the present invention is a method to decrease leptin receptor levels by increasing the breakdown of leptin receptor protein, *i.e.*, by binding of an antibody such that the leptin receptor protein is targeted for removal. Another embodiment is to decrease leptin levels by increasing the synthesis of a soluble form of the leptin receptor, which binds to free leptin.

Another embodiment of the present invention is a method to administer compounds which affect leptin receptor structure, function or homodimerization properties. Such compounds include, but are not limited to, proteins, nucleic acids, carbohydrates or other molecules which upon binding alter leptin receptor structure, function, or homodimerization properties, and thereby render the receptor ineffectual in its activity.

#### **5.5.2. Restoration or Increase in Ob or ObR Expression or Activity to Decrease Bone Mass**

With respect to an increase in the level of normal Ob or ObR gene expression and/or gene product activity, Ob or ObR nucleic acid sequences can be utilized for the treatment of bone disorders. Where the cause of the disorder is a defective Ob or ObR, treatment can be administered, for example, in the form of gene replacement therapy. Specifically, one or more copies of a normal Ob or ObR gene or a portion of the Ob or ObR gene that directs the production of an Ob or ObR gene product exhibiting normal function, may be inserted into the appropriate cells within a patient or animal subject, using vectors which include, but are not limited to, adenovirus, adeno-associated virus, retrovirus and herpes virus vectors, in addition to other particles that introduce DNA into cells, such as liposomes.

Because the ObR gene is expressed in the brain, including the choroid plexus and hypothalamus, such gene replacement therapy techniques involving ObR should be capable of delivering ObR gene sequences to these cell types within patients. Thus, the techniques for delivery of the ObR gene sequences should be designed to readily cross the blood-brain barrier, which are well known to those of skill in the art (see, *e.g.*, PCT application, publication No. WO89/10134, which is incorporated herein by reference in its entirety), or, alternatively, should involve direct administration of such ObR gene sequences to the site of the cells in which the ObR gene sequences are to be expressed. Alternatively, targeted homologous recombination can be utilized to correct the defective endogenous Ob or ObR gene in the appropriate tissue. In animals, targeted homologous recombination can be used to correct the defect in ES cells in order to generate offspring with a corrected trait.

Additional methods which may be utilized to increase the overall level of Ob or ObR gene expression and/or activity include the introduction of appropriate Ob or ObR-expressing cells, preferably autologous cells, into a patient at positions and in numbers which are sufficient to ameliorate the symptoms of bone disorders associated with increased bone mass. Such cells may be either recombinant or non-recombinant. Among the cells which can be administered to increase the overall level of Ob or ObR gene expression in a patient are normal cells, preferably choroid plexus cells, or hypothalamus cells which express the ObR gene, or adipocytes, which express the Ob gene. The cells can be administered at the anatomical site in the brain or in the adipose tissue, or as part of a tissue graft located at a

different site in the body. Such cell-based gene therapy techniques are well known to those skilled in the art, see, *e.g.*, Anderson, *et al.*, U.S. Pat. No. 5,399,349; Mulligan & Wilson, U.S. Pat. No. 5,460,959.

Finally, compounds, identified in the assays described above, that stimulate or enhance the signal transduced by activated ObR, *e.g.*, by activating downstream signaling proteins in the ObR cascade and thereby by-passing the defective ObR, can be used to achieve decreased bone mass. The formulation and mode of administration will depend upon the physico-chemical properties of the compound. The administration should include known techniques that allow for a crossing of the blood-brain barrier.

### **5.5.3. Gene Therapy Approaches to Controlling Ob and ObR Activity and Treating or Preventing Bone Disease**

The expression of Ob and ObR can be controlled *in vivo* (*e.g.* at the transcriptional or translational level) using gene therapy approaches to regulate Ob and ObR activity and treat bone disorders. Certain approaches are described below.

With respect to an increase in the level of normal Ob and ObR gene expression and/or Ob and ObR gene product activity, Ob and ObR nucleic acid sequences can be utilized for the treatment of bone diseases. Where the cause of the bone disease is a defective Ob or ObR gene, treatment can be administered, for example, in the form of gene replacement therapy. Specifically, one or more copies of a normal Ob or ObR gene or a portion of the gene that directs the production of a gene product exhibiting normal function, may be inserted into the appropriate cells within a patient or animal subject, using vectors which include, but are not limited to adenovirus, adeno-associated virus, retrovirus and herpes virus vectors, in addition to other particles that introduce DNA into cells, such as liposomes.

Because the ObR gene is expressed in the brain, including the cortex, thalamus, brain stem and spinal cord and hypothalamus, such gene replacement therapy techniques should be capable of delivering ObR gene sequences to these cell types within patients. Thus, the techniques for delivery of the ObR gene sequences should be designed to readily cross the blood-brain barrier, which are well known to those of skill in the art (see, *e.g.*, PCT application, publication No. WO89/10134, which is incorporated herein by reference in its

entirety), or, alternatively, should involve direct administration of such ObR gene sequences to the site of the cells in which the ObR gene sequences are to be expressed.

Alternatively, targeted homologous recombination can be utilized to correct the defective endogenous Ob or ObR gene in the appropriate tissue; *e.g.*, adipose and brain tissue, respectively. In animals, targeted homologous recombination can be used to correct the defect in ES cells in order to generate offspring with a corrected trait.

Additional methods which may be utilized to increase the overall level of Ob or ObR gene expression and/or activity include the introduction of appropriate Ob or ObR-expressing cells, preferably autologous cells, into a patient at positions and in numbers which are sufficient to ameliorate the symptoms of bone disorders, including, but not limited to, osteopetrosis, osteosclerosis and osteochondrosis. Such cells may be either recombinant or non-recombinant. Among the cells which can be administered to increase the overall level of Ob or ObR gene expression in a patient are normal cells, or adipose or hypothalamus cells which express the Ob or ObR gene, respectively. The cells can be administered at the anatomical site in the adipose tissue or in the brain, or as part of a tissue graft located at a different site in the body. Such cell-based gene therapy techniques are well known to those skilled in the art, see, *e.g.*, Anderson, *et al.*, U.S. Pat. No. 5,399,349; Mulligan & Wilson, U.S. Pat. No. 5,460,959.

## **5.6 Pharmaceutical Formulations and Methods of Treating Bone Disorders**

The compounds of this invention can be formulated and administered to inhibit a variety of bone disease states by any means that produces contact of the active ingredient with the agent's site of action in the body of a mammal. They can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as individual therapeutic active ingredients or in a combination of therapeutic active ingredients. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage administered will be a therapeutically effective amount of the compound sufficient to result in amelioration of symptoms of the bone disease and will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular

active ingredient and its mode and route of administration; age, sex, health and weight of the recipient; nature and extent of symptoms; kind of concurrent treatment, frequency of treatment and the effect desired.

### 5.6.1 Dose Determinations

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the  $LD_{50}$  (the dose lethal to 50% of the population) and the  $ED_{50}$  (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio  $LD_{50}/ED_{50}$ . Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the  $ED_{50}$  with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the  $IC_{50}$  (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Specific dosages may also be utilized for antibodies. Typically, the preferred dosage is 0.1 mg/kg to 100 mg/kg of body weight (generally 10 mg/kg to 20 mg/kg), and if the antibody is to act in the brain, a dosage of 50 mg/kg to 100 mg/kg is usually appropriate. If the antibody is partially human or fully human, it generally will have a longer half-life within the human body than other antibodies. Accordingly, lower dosages of partially human and fully human antibodies is often possible. Additional modifications may be used to further

stabilize antibodies. For example, lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration (*e.g.*, into the brain). A method for lipidation of antibodies is described by Cruikshank et al. ((1997) *J. Acquired Immune Deficiency Syndromes and Human Retrovirology* 14:193).

5 A therapeutically effective amount of protein or polypeptide (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight.

10 Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide or antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antibody, protein, or polypeptide in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5 or 6 weeks.

15 The present invention further encompasses agents which modulate expression or activity. An agent may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e.*, including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

20 It is understood that appropriate doses of small molecule agents depends upon a number of factors known to those of ordinary skill in the art, *e.g.*, a physician. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention. Exemplary  
30 doses include milligram or microgram amounts of the small molecule per kilogram of subject

or sample weight (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram.

### 5.6.2 Formulations and Use

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.* gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use. In general, water, a suitable oil, saline, aqueous dextrose (glucose), and related sugar solutions and glycols such as propylene glycol or polyethylene glycols are suitable carriers for parenteral solutions. Solutions for parenteral administration contain preferably a water soluble salt of the active ingredient, suitable stabilizing agents and, if necessary, buffer substances. Antioxidizing agents such as sodium bisulfate, sodium sulfite or ascorbic acid, either alone or combined, are suitable stabilizing agents. Also used are citric acid and its salts and sodium ethylenediaminetetraacetic acid (EDTA). In addition, parenteral solutions can contain preservatives such as benzalkonium chloride, methyl- or propyl-paraben and chlorobutanol. Suitable pharmaceutical carriers are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection.

Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Additionally, standard pharmaceutical methods can be employed to control the duration of action. These are well known in the art and include control release preparations and can include appropriate macromolecules, for example polymers, polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methyl cellulose, carboxymethyl cellulose or protamine sulfate. The concentration of macromolecules as well as the methods of incorporation can be adjusted in order to control release. Additionally, the agent can be incorporated into particles of polymeric materials such as polyesters, polyamino acids, hydrogels, poly (lactic acid) or ethylenevinylacetate copolymers. In addition to being incorporated, these agents can also be used to trap the compound in microcapsules.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Useful pharmaceutical dosage forms, for administration of the compounds of this invention can be illustrated as follows:

**Capsules:** Capsules are prepared by filling standard two-piece hard gelatin capsules each with the desired amount of powdered active ingredient, 175 milligrams of lactose, 24 milligrams of talc and 6 milligrams magnesium stearate.

**Soft Gelatin Capsules:** A mixture of active ingredient in soybean oil is prepared and injected by means of a positive displacement pump into gelatin to form soft gelatin capsules containing the desired amount of the active ingredient. The capsules are then washed and dried.

**Tablets:** Tablets are prepared by conventional procedures so that the dosage unit is the desired amount of active ingredient. 0.2 milligrams of colloidal silicon dioxide, 5 milligrams of magnesium stearate, 275 milligrams of microcrystalline cellulose, 11 milligrams of cornstarch and 98.8 milligrams of lactose. Appropriate coatings may be applied to increase palatability or to delay absorption.

Injectable: A parenteral composition suitable for administration by injection is prepared by stirring 1.5% by weight of active ingredients in 10% by volume propylene glycol and water. The solution is made isotonic with sodium chloride and sterilized.

Suspension: An aqueous suspension is prepared for oral administration so that each 5 millimeters contain 100 milligrams of finely divided active ingredient, 200 milligrams of sodium carboxymethyl cellulose, 5 milligrams of sodium benzoate, 1.0 grams of sorbitol solution U.S.P. and 0.025 millimeters of vanillin.

Gene Therapy Administration: Where appropriate, the gene therapy vectors can be formulated into preparations in solid, semisolid, liquid or gaseous forms such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, and aerosols, in the usual ways for their respective route of administration. Means known in the art can be utilized to prevent release and absorption of the composition until it reaches the target organ or to ensure timed-release of the composition. A pharmaceutically acceptable form should be employed which does not ineffectuate the compositions of the present invention. In pharmaceutical dosage forms, the compositions can be used alone or in appropriate association, as well as in combination, with other pharmaceutically active compounds.

Accordingly, the pharmaceutical composition of the present invention may be delivered via various routes and to various sites in an animal body to achieve a particular effect (see, *e.g.*, Rosenfeld *et al.* (1991), *supra*; Rosenfeld *et al.*, Clin. Res., 3 9(2), 31 1A (1991 a); Jaffe *et al.*, *supra*; Berkner, *supra*). One skilled in the art will recognize that although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route. Local or systemic delivery can be accomplished by administration comprising application or instillation of the formulation into body cavities, inhalation or insufflation of an aerosol, or by parenteral introduction, comprising intramuscular, intravenous, peritoneal, subcutaneous, intradermal, as well as topical administration.

The composition of the present invention can be provided in unit dosage form wherein each dosage unit, *e.g.*, a teaspoonful, tablet, solution, or suppository, contains a predetermined amount of the composition, alone or in appropriate combination with other active agents. The term "unit dosage form" as used herein refers to physically discrete units

suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of the compositions of the present invention, alone or in combination with other active agents, calculated in an amount sufficient to produce the desired effect, in association with a pharmaceutically acceptable diluent, carrier, or vehicle, where appropriate.

5 The specifications for the unit dosage forms of the present invention depend on the particular effect to be achieved and the particular pharmacodynamics associated with the pharmaceutical composition in the particular host.

Accordingly, the present invention also provides a method of transferring a therapeutic gene to a host, which comprises administering the vector of the present invention, preferably as part of a composition, using any of the aforementioned routes of administration or alternative routes known to those skilled in the art and appropriate for a particular application. The "effective amount" of the composition is such as to produce the desired effect in a host which can be monitored using several end-points known to those skilled in the art. Effective gene transfer of a vector to a host cell in accordance with the present invention to a host cell can be monitored in terms of a therapeutic effect (*e.g.* alleviation of some symptom associated with the particular disease being treated) or, further, by evidence of the transferred gene or expression of the gene within the host (*e.g.*, using the polymerase chain reaction in conjunction with sequencing, Northern or Southern hybridizations, or transcription assays to detect the nucleic acid in host cells, or using immunoblot analysis, antibody-mediated detection, mRNA or protein half-life studies, or particularized assays to detect protein or polypeptide encoded by the transferred nucleic acid, or impacted in level or function due to such transfer).

These methods described herein are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan. Moreover, the effective amount of the compositions can be further approximated through analogy to compounds known to exert the desired effect.

Furthermore, the actual dose and schedule can vary depending on whether the compositions are administered in combination with other pharmaceutical compositions, or depending on interindividual differences in pharmacokinetics, drug disposition, and metabolism. Similarly, amounts can vary in *in vitro* applications depending on the particular cell line utilized (*e.g.*, based on the number of adenoviral receptors present on the cell surface,

or the ability of the particular vector employed for gene transfer to replicate in that cell line). Furthermore, the amount of vector to be added per cell will likely vary with the length and stability of the therapeutic gene inserted in the vector, as well as also the nature of the sequence, and is particularly a parameter which needs to be determined empirically, and can be altered due to factors not inherent to the methods of the present invention (for instance, the cost associated with synthesis). One skilled in the art can easily make any necessary adjustments in accordance with the exigencies of the particular situation.

The following examples are offered by way of example, and are not intended to limit the scope of the invention in any manner.

## 6 EXAMPLES

### 6.1 Generation, Characterization and Treatment of Animals

Breeders and mutant mice (C57BL/6J Lep<sup>ob</sup>, C57BL/6J Lep<sup>db</sup>, C57BL/6J A<sup>y/a</sup>) were purchased from the Jackson Laboratory. Generation of A-ZIP/F-I transgenic mice has been previously reported. (Moitra *et al.*, 1998, *Genes Dev* 12, 3168-3181) Genotyping was performed according to established protocols (Chua *et al.*, 1997, *Genomics* 45, 264-270; Moitra *et al.*, 1998, *Genes Dev* 12, 3168-3181; Nanae *et al.*, 1998, *Lab Animal Sci* 48, 103-104). Animals were fed a regular diet (Purina #5001) or, when indicated, a high fat/high carbohydrate diet (Bio-serv # F3282). Bone specimens were processed as described (Ducy *et al.*, 1999, *Genes Dev* 13, 1025-1036).

### 6.2 Demonstration of Bone Mass Phenotype in ob/ob and db/db Mice

Hypogonadism induces an increase in osteoclast number and in bone resorption activity which leads to a low bone mass phenotype (Riggs and Melton, 1986, *N Engl J Med* 314, 1676-1678). Thus, the ob/ob mice that have a hypogonadism of hypothalamic origin should have a lower bone mass than wild-type littermates (Ahima *et al.*, 1996, *Nature* 382, 250-252; Chehab *et al.*, 1996, *Nat Genet* 12, 318-320; Ahima *et al.*, 1997, *J Clin Invest* 99, 391-395). To determine if the obesity of the ob/ob mice could affect their expected low bone mass phenotype, X-ray analysis of vertebrae and long bones of 6-month-old wild-type and ob/ob mice was performed using a Faxitron (Phillips). Surprisingly, the bones of the ob/ob mice appeared much denser than those of their wild-type littermates (Figure 1A),

demonstrating the presence of a higher amount of mineralized bone matrix. Given the poor sensitivity of X-rays to quantify bone mass abnormalities, the increase in bone density was an indication of a major change in bone architecture (*i.e.* affecting more than 30% of the bone matrix).

5           Histologic analysis was performed on undecalcified sections stained with the von Kossa reagent and counterstained with Kernechtrot. In 3 and 6 month-old mice the presence of many more thick trabeculae in the bones of ob/ob mice compared to those of wild-type mice was observed (Figure 1B). The cortical bone was not affected. Histomorphometric quantification according to standard techniques were performed (Parfitt *et al.*, 1987, J Bone  
10   Min Res 2, 595-610) using the Osteomeasure Analysis System (Osteometrics, Atlanta). Statistical differences between groups (n=4 to 6) were assessed by Student's test. The experiments showed a nearly 2-fold increase in trabecular bone volume in long bones and vertebrae of ob/ob mice compared to wild-type littermates (Figure 1 C). This phenotype was observed in both sexes. The functional consequences of this increase in bone mass were  
15   analyzed by comparing the biomechanical properties of long bones of 6 month-old ob/ob mice, wild-type mice, and wild-type mice that have been ovariectomized (wild-type-ovx) for 4 months to mimic the hypogonadic state of the ob/ob mice. An assay in which femora were tested to failure by three-point bending on a servo-hydraulic testing machine (Zwick GmbH & Co.) at a constant displacement rate of 10 mm/min was used to determine failure load, which  
20   is a measure of the strength of the bones. Failure load of the bones from wild-type and ob/ob mice were undistinguishable but significantly higher than the one observed in the bones of wild-type-ovx mice (Figure 1D). This result indicates that leptin deficiency has a beneficial effect on the biomechanical properties of the bones. Analysis of the bones of the db/db mice that have an inactivating mutation of the leptin receptor was also performed (Tartaglia *et al.*,  
25   1995, Cell 83, 1263-1271). Like the ob/ob mice, the db/db mice are obese and hypogonadic. There was an increase in the number of trabeculae in both long bones and vertebrae similar to that observed in ob/ob mice (Figure 1E) resulting in a 3-fold increase in bone volume compared to wild-type mice (Figure 1F). This latter result demonstrates genetically that leptin signals through its known receptor to affect bone mass.

### 6.3 The High Bone Mass Phenotype of the ob/ob and db/db Mice is Secondary to the Absence of Leptin Signaling and Not to Obesity

The high bone mass phenotype of the ob/ob and ob/db mice could be secondary either to a lack of leptin signaling or to the obesity of these mice. To distinguish between these two possibilities, several additional groups of mutant mice were analyzed for high bone mass phenotype as in Example 2. First, a low fat diet which postpones the appearance of obesity in ob/ob mice was fed to ob/ob mice, and they had a normal weight at one month of age. However, the mice already had a high bone mass phenotype at that age (Figure 2A). Second, heterozygote leptin-deficient mice (ob/+) that are not obese were analyzed. These animals also had a high bone mass phenotype (Figure 2B). Third, the bones of other mouse models of obesity that are not primarily related to leptin signaling were observed. Another genetic model of obesity, the Agouti yellow (A<sup>y/a</sup>) mice (Herberg and Coleman, 1977, Metabolism 26, 59-99), had a normal bone mass, as did wild-type mice fed with a high fat diet (Figures 2C and 2D).

Thus, the existence of a high bone mass phenotype in ob/ob mice prior to the appearance of obesity and in ob/+ mice that are not obese, and its absence in leptin-unrelated models of obesity demonstrate that it is the absence of leptin signaling, not the obesity, that causes this high bone mass phenotype.

### 6.4 Increased Osteoblast Function in ob/ob and db/db Mice

The increase in bone mass could be due to an increase in osteoblastic bone formation, to a decrease in osteoclastic bone resorption, or to a combination of both abnormalities. To study osteoblast function *in vivo*, the rate of bone formation was quantified following double labeling with calcein, a marker of newly formed bone (Figure 3A) as described (Ducy *et al.*, 1999, Genes Dev 13, 1025-1036). Calcein was injected twice at 8 day intervals and animals were sacrificed two days later. In 3 month-old, and 6 month-old ob/ob mice there was a 70% and 60%, respectively, increase of the bone formation rate compared to the one of wild-type littermates (Figure 3B). This result demonstrated that the high bone mass phenotype of the ob/ob mice was due, at least in part, to an increase in bone formation activity. Remarkably, considering the massive increase of the bone formation rate, the surface of the osteoblasts as well as the osteoblast number were not increased in ob/ob mice, indicating that leptin

deficiency affects the function of the osteoblasts and not their differentiation after birth (Figure 3C). Likewise, calcein labeling of the db/db mice showed an increase in the rate of bone formation in both long bones and vertebrae in the face of a normal number of osteoblasts (Figures 3D and 3E). As expected given the existence of the hypogonadism, the number of osteoclasts was increased in both mutant mouse strains (Figure 3F) suggesting that the high bone mass phenotype of the ob/ob and db/db mice may have developed despite an increase in bone resorption.

The specificity of the effect of the absence of leptin signaling on osteoblast function using the same groups of control animals as above was determined. One month-old ob/ob animals fed a low fat diet and heterozygote leptin-deficient mice, both of which were lean, also had a significant increase in their rate of bone formation (Figure 3G). In contrast, Ar/a mutant mice as well as wild-type mice fed a high fat diet, two leptin-unrelated models of obesity, had normal bone formation parameters (Figure 3H).

#### 6.5 Analysis of Osteoclast Function in ob/ob Mice

The coexistence of a high bone mass phenotype and of hypogonadism was so exceptional that it raised the hypothesis that bone resorption might be defective in these mice. The increased urinary elimination of deoxypyridinoline crosslinks (Dpd), a biochemical marker of bone resorption (Eyre *et al.*, 1988, *Biochem* 252, 494-500), in the ob/ob mice argued against this hypothesis (wild-type:  $10.5 \pm 2.5$  nM Dpd/mM creatinine; ob/ob:  $24.0 \pm 4.0$  nM Dpd/mM creatinine). Deoxypyridinoline crosslinks were measured in morning urines using the Pylinks-D immunoassay kit (Metra Biosystem). Creatinine values were used for standardization between samples (Creatinine kit, Metra Biosystem). Circulating concentration of  $17\beta$ -estradiol and leptin were quantified by radioimmunoassays, using the third generation estradiol kit (Diagnostic system laboratories) and the mouse leptin RIA kit (Linco), respectively. Estradiol is an estrogen analog and also a Selective Estrogen Receptor Modulator (SERM). Coadministration of an estrogen analog with an Ob or ObR inhibitor further increases the high bone mass phenotype.

Nevertheless, to address this point more thoroughly the hypogonadism of the ob/ob mice was exploited. Hypogonadism normally leads to an increase in osteoclasts number and in bone resorptive activity. Thus, if the osteoclasts of the ob/ob mice were functional,

correcting the hypogonadism of these mice should decrease their rate of bone resorption by diminishing the number of osteoclasts and thereby should further increase their bone mass. On the other hand, if the osteoclasts of the ob/ob mice were not functioning properly, correcting their hypogonadism should not affect the severity of their high bone mass phenotype. To determine which of these two possibilities was correct, 17  $\beta$ -estradiol or placebo pellets (Innovative Research of America) were implanted subcutaneously in 2-month-old female ob/ob mice to maintain a serum concentration of 250 pg/mL. These animals were analyzed after a 3-month treatment period.

As expected, the estradiol treatment corrected their hypogonadism as judged by the aspect of their uteri and their levels of estradiol in blood (Figure 4A), and furthermore resulted in a normalization of the osteoclast number (Figure 4C). It also led to a further increase of the high bone mass phenotype of these mice compared to placebo treated ob/ob mice, thus eliminating a defect of bone resorption as the origin of the high bone mass phenotype in ob/ob mice (Figure 4B). Estradiol-treated ob/ob mice had a 50% increase in bone volume compared to estradiol-treated wild-type mice and a 3-fold increase compared to untreated wild-type mice at the end of the treatment period (Figure 4D). Similar results were obtained in male ob/ob mice treated with testosterone implants. Finally, the function of the osteoclasts of ob/ob and db/db mice was studied *in vitro* in an assay using hematopoietic progenitor cells from wild type, ob/ob, or db/db mice and growth factors known to induce the differentiation of these cells into functional osteoclasts. Mouse osteoclasts were generated *in vitro* according to protocols previously reported (Simonet *et al.*, 1997, Cell 89, 309-319; Quinn *et al.*, 1998, Endocrinology 139, 4424-4427). Bone-marrow cells of wt, ob/ob, and db/db mice were cultured at an initial density of  $10^6$  cells/well on 24-well tissue culture plates or dentin chips in  $\alpha$ -MEM (Sigma) containing 10% FBS (Hiclon), murine M-CSF 40 ng/mL (Sigma), RANKL/ODF 25 ng/mL (Peprotech),  $10^{-8}$  M dexamethasone (Sigma) and  $10^{-8}$  1,25 dihydroxy vitamin D3. Medium was changed every other day. After 6 days of culture, cells were fixed in 3.7% formalin. Osteoclasts formed on plastic plates were stained for tartrate resistant acid phosphatase. Cells on dentin chips were removed by treatment with sodium hypochloride solution. Dentin chips were then stained with toluidine blue to visualize resorption pits.

As shown in Figure 4E, wild-type, *ob/ob*, and *db/db* hematopoietic progenitor cells differentiated equally well into osteoclasts able to resorb a matrix.

Thus, these experiments demonstrate that there is no detectable functional defect of the osteoclasts in *ob/ob* and *db/db* mice and establish that the high bone mass phenotype of these mouse mutant strains results exclusively from the increase in bone formation secondary to the absence of leptin signaling.

## 6.6 Absence of Leptin Signaling in Osteoblasts

The previous analyses indicate that leptin is an inhibitor of osteoblastic bone formation. Moreover, the existence of a high bone mass phenotype in *db/db* mice demonstrates that leptin must bind to its known receptor to fulfill this function. In theory, leptin could either act directly on osteoblasts, indirectly through the release of a second factor present in fat, or by using a hypothalamic pathway as it does for the control of body weight. These three possible mechanisms of action were tested.

Expression of leptin in osteoblasts was studied in subconfluent primary osteoblast cultures from wild-type mice which were maintained for 4 days in 10% FBS mineralization medium and then subsequently switched to 0.5% for 2 days and replaced daily. Medium was replaced 2 hours before a 20 min treatment with 80 ng/mL leptin (Sigma) or 40 ng/mL Oncostatin M (R&D Diagnostics) or vehicle. RNA extractions were performed using Triazol (Gibco). Northern blots were performed with 15  $\mu$ g of total RNA according to methods well known in the art.

Expression of leptin in osteoblasts could not be detected even after a long film exposure, indicating that an autocrine regulation was unlikely (Figure 5A). Leptin expression could also not be detected in whole bone samples, providing an indirect argument against a paracrine regulation of osteoblast function by leptin (Figure 5A). In any case, a paracrine and/or an endocrine regulation of osteoblast function by leptin would require that functional leptin receptors are present on osteoblasts. There are several transcripts of the leptin receptor, but only one, *Ob-Rb*, is thought to have signal transduction ability (Tartaglia *et al.*, 1995, Cell 83, 1263-1271; Chen *et al.*, 1996, Cell 84, 491-495; Lee *et al.*, 1996, Nature 379, 632-635).

The expression of this transcript of leptin receptor is highly, although not strictly, hypothalamus-specific. RT-PCR experiments were performed to search for *Ob-Rb* transcripts

5 in primary osteoblasts and whole bone samples. RT-PCR analysis (27 cycles) of *Ob-Rb* expression was performed on random-primed cDNAs using the following primers: 5'-TGGATAAACC CTTGCTCTTCA-3', and 5'-ACACTGTTAATTTACACCAGAG-3' (Friedman and Halaas, 1998, *Nature* 395, 763-770). Amplification of *Hprt* was used as an internal control for cDNA quality using the following primers: 5'-GTTGAGAGATCATCTCCACC-3', and 5'-AGCGATGATG AACCAGGTTA-3'.

In several experiments using a number of amplification cycles necessary to detect *Ob-Rb* transcripts in hypothalamus, there was no detection of *Ob-Rb* expression in calvaria, long bone, and primary osteoblast cultures (Figure 5B).

10 To determine whether or not leptin could transduce its signal in osteoblasts, serum-starved primary osteoblasts isolated from wild-type mice were treated with leptin. Primary osteoblast cultures from calvaria of newborn wild-type or db/db mice were established as previously described (Ducy *et al.*, 1999, *Genes Dev* 13, 1025-1036) and maintained in mineralization medium ( $\alpha$ MEMI 0.1 mg/mL ascorbic acid; 5 mM  $\alpha$ -glycerophosphate) supplemented with 10% PBS. Cultures of mutant cells were maintained in this medium for 15 days before analysis. Cultures derived from wild-type mice were maintained for 10 days in this medium then the percentage of serum was reduced to 0.5% and the medium supplemented with 1.2  $\mu$ g/mL leptin (Sigma) or vehicle for 5 days.

20 The phosphorylation of Stat3, a downstream effector of leptin signaling in its target cells (Tartaglia *et al.*, 1995, *Cell* 83, 1263-1271; Baumann *et al.*, 1996, *Proc Natl Acad Sci USA* 93, 8374-8378; Ghilardi *et al.*, 1996, *Proc Natl Acad Sci USA* 93, 6231-6235; Vaisse *et al.*, 1996, *Nat Genet* 14, 95-97), was monitored, in addition to the expression of two immediate early genes whose transcription is increased following leptin treatment of target cells (Elmqvist *et al.*, 1997, *Endocrinology* 138, 839-842). As a positive control oncostatin-M that induces Stat3 phosphorylation and activates the expression of the same two immediate early genes in osteoblasts (Levy *et al.*, 1996, *Endocrinology* 137, 1159-1165) was utilized. Western blot analysis was performed to determine Stat3 phosphorylation as follows. Cells were lysed, protein extracts were separated on a 7.5% SDS-PAGE and blotted on nitrocellulose (Biorad) for immunoblotting assay by methods well known in the art. Analysis of Stat3 phosphorylation was performed using the PhosphoPlus Stat3 (Tyr705) Antibody kit (New England Biolabs) according to the manufacturer's instructions.

As shown in Figure 5C, treatment of osteoblasts with Oncostatin-M did induce Stat3 phosphorylation while leptin treatment did not. Several doses of leptin, physiologic and supraphysiologic, were used in this experiment, yet all of them failed to induce Stat3 phosphorylation. Similarly, expression of *Tis11* and *c-fos*, analyzed by standard Northern analysis methods, was quickly and transiently activated by oncostatin-M but not by leptin (Figure 5D). Finally, the effect of a long-term leptin treatment of primary osteoblast cultures from wild-type mice on extracellular matrix synthesis and bone matrix mineralization was determined. The presence of a collagen-rich extracellular matrix and of mineralization nodules was assessed by whole-mount staining of the cultures by the van Gieson and von Kossa reagent, respectively. No difference was observed when assessing collagen synthesis or mineralization nodule formation between control and leptin-treated cultures (Figure 5E).

Finally, if there is no functional leptin receptor on osteoblasts, then wild-type and *db/db* osteoblasts should be undistinguishable in *ex vivo* culture. Primary cultures of osteoblasts from *db/db* and wild-type mice were analyzed for their ability to generate a bona fide bone extracellular matrix and to mineralize it. For all the parameters analyzed, which were alkaline phosphatase staining, type I collagen production and formation of mineralization nodules, there was no difference between wild type and *db/db* primary osteoblast cultures (Figure SF). Taken together, these results indicate that leptin action on bone formation in the entire animal does not require leptin binding to a receptor located on the osteoblasts.

## 6.7 High Bone Mass in Absence of Fat Tissue

To address the possibility that this action of leptin could require the presence of fat, a transgenic mouse model expressing, exclusively in adipocytes, a dominant negative protein termed A-ZIP was utilized (Moitra *et al.*, 1998, *Genes Dev* 12, 3168-3181). This dominant negative protein abolishes the DNA-binding ability of most B-ZIP transcription factors, a class of transcription factors critical for adipocyte differentiation. As a result the A-ZIP/F-1 transgenic mice have no white adipose tissue, which is the type of fat regulated by leptin signaling, and dramatically reduced amounts of inactive brown adipose tissue. They also have a 20-fold reduction in leptin synthesis (Moitra *et al.*, 1998, *Genes Dev* 12, 3168-3181). In A-

ZIP/F-I transgenic mice the same high bone mass phenotype due to an increase in osteoblast function was observed as is seen in ob/ob and db/db mice (Figure 6).

This experiment has two implications. First, it confirms that leptin deficiency, not high fat index, is responsible for the high bone mass phenotype of the ob/ob and db/db mice. Second, it demonstrates that fat tissue is not a necessary relay for the action of leptin on bone formation.

#### 6.8 Intracerebroventricular Infusion of Leptin Corrects the High Bone Mass Phenotype of the ob/ob Mice

Lastly, the issue of whether leptin binding to its hypothalamic receptor could correct the high bone mass phenotype of the ob/ob mice as it can rescue their obesity phenotype was addressed. To that end pumps delivering either PBS or leptin (8 ng/hr) in the third ventricle of ob/ob mice were inserted as follows. Animals were anesthetized with avertin and placed on a stereotaxic instrument (Stoelting). The calvaria was exposed and a 0.7 mm hole was drilled upon bregma. A 28-gauge cannula (Brain infusion kit U, Alza) was implanted into the third ventricle according to the following coordinates: midline, -0.3 AP, 3 mm ventral (0 point bregma). The cannula was secured to the skull with cyanoacrylate, and attached with Tygon tubing to an osmotic pump (Alza) placed in the dorsal subcutaneous space of the animal. The rate of delivery was 0.25  $\mu$ l/hour (8 ng/hr of leptin (Sigma)) or PBS for 28 days. The dosage has been previously shown to have no effect when administered systemically (Halaas *et al.*, 1997, Proc Natl Acad Sci USA 94, 8878-8883). To be as close as possible to the biological situation of the ob/ob mice, the animals in which pumps were inserted were ovariectomized to avoid any artificial increase in bone mass due to the correction of their hypogonadism. The pumps were left in place for 28 days and double-labeling with calcein was performed to measure the bone formation parameters.

Classical histology showed that the bone of leptin-treated mice but not of the PBS-treated mice had regained a normal appearance (Figure 7A). They had fewer trabeculae and these trabeculae looked more regular than in the PBS-treated ob/ob mice. Bone volume, trabeculae thickness and bone formation rates were all significantly decreased in leptin-treated mice (Figure 7A). No leptin in the serum of these animals was detected using a specific radioimmunoassay. The rescue of the bone phenotype by leptin

intracerebroventricular infusion, together with the absence of measurable circulating leptin, demonstrates that control of bone formation is a neuroendocrine leptin-dependent function.

All patents and publications mentioned in the specifications are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art readily appreciates that the patent invention is well adapted to carry out the objectives and obtain the ends and advantages mentioned as well as those inherent therein. Leptin, leptin receptor, leptin antibodies, leptin analogs, leptin antagonists, pharmaceutical compositions, treatments, methods, procedures and techniques described herein are presently representative of the preferred embodiments and are intended to be exemplary and are not intended as limitations of the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the pending claims.